Studies on the transfer and refolding of lysozyme in reversed micelles

Mall, Sanjay

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STUDIES ON THE TRANSFER AND REFOLDING OF LYSOZYME IN REVERSED MICELLES

submitted by

SANJAY MALL

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

This thesis is dedicated to my father

Dr M. P. Mall

for being the model of

diligence and dedication to one's work.

ABSTRACT

The aim of this study was to investigate the factors affecting the use of reversed micelles to refold denatured lysozyme. Reversed micelles are aggregates of surfactant molecules in organic solvents which are thermodynamically stable. The partitioning of native and non-native lysozyme (1 mg/ml) into AOT/isooctane reversed micelles has been studied. The effect of ionic strength, pH, surfactant type and its concentration, denaturant type and its concentration were investigated on the partitioning behaviour of native lysozyme. Electrostatic interactions were found to be the driving force for solubilisation of native lysozyme into reversed micelles. Structural studies using CD and fluorescence show lysozyme to be completely unfolded at concentrations of 6 M GuHCl. Prior to refolding, lysozyme in 6 M GuHCl had to be partitioned into reversed micelles. However, partitioning of non-native, non-reduced lysozyme into the micelles occurred at a maximum concentration of 1.5 M GuHCl. By reducing the protein, this maximum GuHCl concentration was increased to 3.2 M. As indicated by conformational studies lysozyme was transferred into the micelles in a partially denatured form. Investigations were performed using urea as the denaturant but it was found to be unsuitable for transfer of lysozyme into micelles. Mixed surfactant micelles were made by combining AOT with the nonionic surfactants Tween 85 and Tween 20 in an attempt to improve the transfer step. However, it was found that the transfer of lysozyme with mixed surfactants occurred at even lower concentrations of GuHCl than when AOT was used alone. This was attributed to smaller size of micelles formed by these nonionic surfactants as measured by Karl-Fischer titration. A denaturant system consisting of mixtures of GuHCl and urea at different molar ratios (1:4, 0.5:4.5, 0.3:4.7) was successful in transferring lysozyme at much higher denaturant concentrations than was achieved with GuHCl. Fluorescence and CD data also show that the unfolding power of these mixtures is greater than urea but not as effective as GuHCl. Non-native, non-reduced lysozyme was shown to regain full activity after a 100-fold dilution. Refolding reduced lysozyme in an aqueous solution gave an optimum recovery of 65%. The protein did not give a 100% yield due to the competing aggregation reaction. After the transfer of non-reduced, non-native lysozyme into reversed micelles using GuHCl and mixtures of GuHCl/urea, recovery of the protein in the aqueous phase resulted in 100% activity. The refolding of reduced lysozyme in reversed micelles did not occur under any of the conditions investigated. This resulted in the formation of a protein/surfactant complex consisting of the majority of the protein with 2-5% AOT.

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NOMENCLATURE

[ ]	Concentration	mol dm^{-3} or M
A	Surface area of reversed micelle water cores	nm^2
a_h	Surfactant head group area	nm^2
a_{surf}	Surfactant head group area	nm^2
A_{280}	Absorbance at 280 nm	-
A_{450}	Absorbance at 450 nm	-
AOT	Aerosol-ot (Sodium di-2-ethylhexyl sulfosuccinate)	-
C	Protein concentration	mg/ml
C_{aq}	Concentration of lysozyme in the aqueous phase	mg/ml
CD	Circular dichroism	-
C_i	Concentration of lysozyme at the interface	mg/ml
C_{lys}	Total lysozyme concentration	mg/ml
CMC	Critical micelle concentration	-
C_{rm}	Concentration of lysozyme in the organic phase	mg/ml
CTAB	Cetyltrimethylammonium bromide	-
d	Pathlength	cm
DLS	Dynamic light scattering	-
e	Electronic charge	-
GuHCl	Guanidine hydrochloride	-
I	Ionic strength	M
k	Boltzman constant	-
K^{-1}	Debye length	Å
KCl	Potassium chloride	-
l	Length of fully extended hydrocarbon tail	nm
M_r	Molecular weight	Da
N_A	Avogadro number	-
NaCl	Sodium chloride	-

N_m	Number of reversed micelles per litre of the total system	-
o/w	Oil-in-water microemilsion	-
$pH_{optimum}$	Optimum pH for protein solubilisation	-
pH_{st}	pH prior to phase equilibration	-
pH_{wp}	pH inside reversed micelles	-
pI	Isoelectric point	-
rpm	Revolutions per minute	-
r	Radius	nm or m
RNase	Ribonuclease A	-
T	Absolute temperature	K
TOMAC	Trioctylmethylammonium chloride	-
UV	Ultraviolet spectroscopy	-
v	Volume of hydrophobic tail	nm^3
V	Volume of water pools	dcm^{-3}
V_{aq}	Volume of the aqueous phase	ml
V_r	Phase volume ratio, V_{aq}/V_{rm}	-
w/o	Water-in-oil microemulsion	-
Wo	Molar ratio, $[H_20]/[Surfactant]$	-
Z_i	Charge on ion	-
Greek		
ε	Dielectric constant	-
ε_{aq}	Molar extinction coefficient in the aqueous phase	$cm^{-1}M^{-1}$
ε_{org}	Molar extinction coefficient in the organic phase	$cm^{-1}M^{-1}$
ε_{280}	Molar extinction coefficient at 280 nm	$cm^{-1}M^{-1}$
Θ	Molar ellipticity	deg cm^2 $dmol^{-1}$
µg/ml	Micrograms per ml	-

CHAPTER 1
INTRODUCTION

The protein folding problem has been studied for the last 30 years and is still a major obstacle for the production of recombinant proteins in the biotechnology industry. Since the advent of genetic engineering, the production of biologically active mammalian proteins from microbial cells is a routine procedure. Genetic engineering techniques allow one to identify and specifically extract mammalian genes coding for a desired protein. These genes may be cloned into the bacteria by means of a plasmid , a circular piece of DNA containing the inserted gene and the information required for gene functionality in the host organism. The gene is then transcribed and the mRNA is translated to give the desired gene product.

Escherichia coli, a gram negative bacteria produces recombinant proteins as solid intracellular protein aggregates (inclusion bodies) at high expression levels. The genetics of *E. coli* are well understood and it is likely to remain a host for sometime to come. The problem of obtaining high yields of recombinant proteins from bacteria is the formation of inclusion bodies (Section 2.1.2). Inclusion bodies are formed because the environment in the bacterial cell is not ideal for the refolding of mammalian proteins and intermediates are prevented from continuing along the folding pathway to form the native configuration. The production of inclusion body proteins from bacteria is easily performed by fermentation at a low cost. Bacteria grow much faster than mammalian cells and therefore the fermentation times are drastically reduced. Apart from the easy manipulation and large quantities produced the isolation, solubilization and renaturation of the inclusion body protein are relatively simple processes which may be adapted to large scale production. The market for products manufactured via a refolding route is greater than $600M and has been predicted to rise to $5000M by the year 2000 (Thatcher and Hitchcock, 1995). Provided that the molecular structure of the renatured, recombinant protein is identical to its natural counterpart i.e. no differences are observed in amino acid sequences or side chain modifications, structural and functional differences are not usually detectable (Jaenicke, 1990).

However, the major problem that must be overcome is that, in many cases, instead of obtaining the refolded protein an insoluble and inactive aggregate is formed. This aggregation reaction is a side reaction in the folding reaction and occurs due to hydrophobic interactions between partially folded intermediates. This reaction is multimolecular and therefore concentration dependent. This aggregation reaction may be limited by working at low protein concentrations. Currently pharmaceutically important proteins are refolded on the micromolar scale which results in low overall yields. There is therefore a major challenge in this area of biotechnology to refold proteins at higher concentrations than can be achieved at present.

The results of this thesis have been described in the following chapters:

Chapter 1 is a brief introduction to the project.

Chapter 2 contains a review of the published literature with regards to protein folding and reversed micelles.

Chapter 3 describes the experimental procedures and methods of analysis used in this work.

Chapter 4 presents data on the transfer of native, non-native (non-reduced) lysozyme and non-native (reduced) lysozyme into AOT/isooctane reversed micelles. Factors controlling these processes have been investigated. The transfer of lysozyme in urea into reversed micelles has also been investigated.

Chapter 5 describes two novel procedures that have been developed in order to attempt to partition unfolded protein at higher denaturant concentrations. The first one utilises a mixed micellar system consisting of AOT and Tween 85 or Tween 20 and the second uses mixtures GuHCl and urea in different molar ratios.

Chapter 6 reports studies on the backward transfer process involved to recover the protein from micelles. The factors which influence refolding of lysozyme in an aqueous environment have also been investigated. Results have also been presented on attempts on refolding lysozyme inside 50 mM AOT/isooctane reversed micelles.

Finally, in Chapter 7, conclusions are drawn from results presented in previous chapters regarding the partitioning of native and non-native lysozyme and also the refolding studies. Also the suggestions for future work in this area have been included.

CHAPTER 2
LITERATURE REVIEW

This chapter has been divided into two sections. The first one describes the current literature on protein refolding. The second section describes the formation and general properties of reversed micelles together with certain aspects of protein partitioning.

2.1 Protein refolding

For biological activity proteins need to adopt specific three-dimensional folded structures. However, the genetic information for a protein comes from its primary structure of amino acids in its peptide backbone. Many proteins can refold *in vitro* after being completely unfolded therefore, the three-dimensional structure must be determined from the primary structure. How this happens has become to be known as the protein folding problem (Creighton, 1990 and 1992).

Creighton (1990) has broken this problem down into three questions. What kinetic pathway does the protein adopt its native folded conformation? What is the physical basis of the stability of folded conformations? Why does the amino acid sequence determine the folded three dimensional structure and not any other? The ability to predict the folded three-dimensional structure from the amino acid sequence could be answered if one fully understood the above three questions.

Ever since the work of Anfinsen and Haber (1961) and Anfinsen (1973) on the refolding of reduced RNase there has been on-going research in the area of protein folding. To date there are still many questions that need to be addressed and answered. In biotechnology the problem is how to refold a protein reproducibly and in high yield *in vitro*. There still exists no protocol for refolding proteins at high concentrations which leads one to the conclusion that protein folding is an extremely complex procedure. This has recently reviewed by (Creighton (1978), Creighton (1990), Hlodan *et al.* (1991) and Lecomte and Mathews (1993)).

The following sections describe the importance of refolding and the production of recombinant proteins, the refolding problem and the current state of protein refolding and finally methods of improving protein refolding.

2.1.1 The importance of refolding

In the past protein folding was studied purely as an academic research interest. However, its importance in biotechnology for the production of health care products involving refolding steps is greater than $600M and estimated to rise to $5000M by the year 2000 (Thatcher and Hitchcock 1995). This requirement for refolding arises from advances in molecular biology which has enabled the production of biologically active mammalian proteins from microbial cells. The production of biologically active mammalian proteins from microbial cells is now a routine procedure. The bacterium *Escherichia coli* produce solid intracellular protein aggregates at high expression levels. These inclusion bodies contain the recombinant protein in a non-native and non-active form. This production of inclusion body proteins from bacteria have the advantage that they are easily fermented at a low cost. The inclusion body phenomenon and recovery of the protein into its active state will be discussed in the following sections.

2.1.2 Inclusion bodies

The expression of recombinant proteins in bacteria, often results in the formation of inactive protein that accumulates intracellularly. The formation of inactive proteins in bacterial systems appears to be independent of the type of protein (Marston *et al.*, 1986). These large, inactive conglomerates are known as inclusion bodies or refractile bodies and accumulate within the host cell. *Escherichia coli*, can be easily manipulated and grown in large fermenters using inexpensive culture media. It eventually became clear that the expression of a polypeptide did not necessarily include the formation of a native, active protein. A review by Schein, (1989) gives a summary on what is known about inclusion bodies and ways of

increasing the production of soluble protein in bacterial systems. Mitraki *et al.* (1989) have reviewed protein folding intermediates and inclusion body formation.

Electron microscopy analysis shows inclusion bodies as large, spherical particles which are clearly separated from the cytoplasm, but not enclosed by membranes. Inclusion bodies may occupy a large part of the host cell, sometimes spanning the entire diameter of the cell. The size and density of γ-interferon (0.81μm) and prochymosin (1.28μm) based inclusion bodies have been reported by Taylor *et al.* (1986). Bowden *et al.* (1991) have studied the structure and morphology of β-lactamase inclusion bodies produced in *Escherichia coli.* Electron microscopy studies of the highly purified protein aggregates indicated that the periplasmic inclusion bodies were amorphous whereas those in the cytoplasm were highly regular. There are two general categories of inclusion bodies (Vicik, 1990). They can appear as paracrystalline arrays where the protein is presumed to be in a stable, but not necessarily native confirmation or they may be amorphous aggregates of partly and completely denatured heterologous proteins. Inclusion bodies are stabilized by unspecific (hydrophobic) interactions.

The production of recombinant proteins as inclusion bodies has its advantages. The protein is present as a solid in a highly purified, and concentrated state. Purification steps involve cellular disruption and chemical/physical separation and these processes are considered to be relatively simple (Vicik, 1990). Furthermore there may be reduced purification requirement which saves time and reduces yield losses.

To obtain the functional protein from the inclusion bodies, it is usually necessary to dissolve the inclusion bodies in a denaturing medium and then submit the solubilized protein to a renaturation procedure (Kane and Hartley, 1988; Rudolph, 1990).

2.1.3 Recovery of active protein from inclusion bodies

The downstream processing of recombinant proteins obtained by microbial fermentation is outlined in Figure 2.1. To obtain reasonable yields of active protein each of the steps must be carefully optimized. These individual steps are discussed briefly below. To obtain proteins in an active form, the inclusion bodies must be separated from the other cellular components. The cells are lysed using mechanical methods such as homogenisation or by chemical and enzymatic methods. After homogenization, the solids consist of inclusion body and cell debris. The first step in the purification of an intracellular enzyme is the removal of the cell debris. The cell debris is slightly denser than inclusion bodies and can therefore be separated by centrifugation.

After the isolation of inclusion bodies the polypeptides must be solubilized. This step usually requires denaturation by reagents such as guanidine hydrochloride (GuHCl) or urea. Usually 6M GuHCl or 6-8M urea are used. GuHCl is preferred to urea which may contain cyanate ions that can irreversibly modify the protein. When inclusion bodies are formed, incorrect disulphide bonds may be formed. These disulphide bonds may be broken by using reagents such as 2-mercaptoethanol as well as dithiothereitol which reduce proteins and prevent the formation of incorrect disulphide bridges (Fischer *et al.*, 1992).

The renaturation or folding of proteins with correct disulphide bond formation includes both the regeneration of the native, non-covalent interactions and the formation of covalent chemical bonds. The number of possible conformations increases rapidly with the number of cysteine residues that are available for pairing.

Anfinsen (1973), showed that ribonuclease A regenerated the correct native disulphide bonds. With this protein only 1 out of the 105 possible combinations

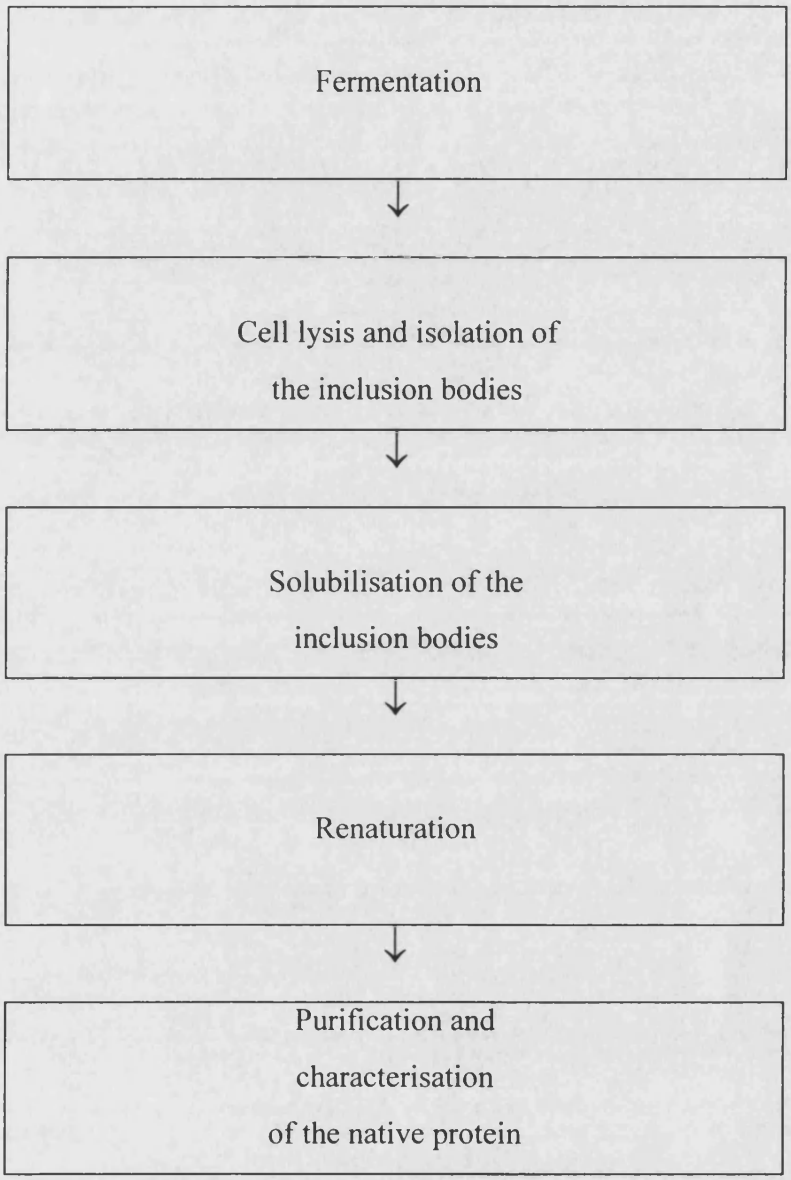

Figure 2.1 Individual steps in obtaining pure protein from inclusion bodies

gives the correct native pairing of the 4 disulphide bonds. Disulphide bonds can be reformed under oxidizing conditions. Air oxidation of reduced cysteine residues can be catalysed in the presence of heavy metal ions, but this method is slow and the yields are low (Chaudhuri, 1993). Saxena *et al.* (1970), have shown that oxidative regeneration systems composed of low molecular weight thiols in the reduced and oxidized forms have regenerated the activity of reduced lysozyme to 60-85% in 30 minutes. This is commonly known as "oxido-shuffling". Previously Epstein and Golberger (1963), showed how thiols assisted the renaturation of reduced lysozyme. The native structure of a protein can be obtained by refolding it in an environment where incorrect disulphide bonds can rearrange to give the correct pairings. The native conformation can be obtained by firstly removing excess denaturants and reducing agents. In addition oxidation of the cysteine residues must be carried out to form the correct disulphide bonds. The denaturant removal can be carried out by dilution of the protein directly into the refolding buffer (Buchner *et al.*, 1991a).

Ideally, the renaturation procedures used both in research and industrial applications would use high protein concentrations. Renaturation processes of non recombinant proteins show that the final yield of renatured protein decreases, sometimes dramatically with increasing concentrations of solubilized protein undergoing renaturation, hence the percentage of insoluble protein aggregates increases. Because of this problem, refolding of non-recombinant proteins must be performed at low protein concentrations (1 to 20 μg/ml), (Goldberg *et al.*, 1991). Aggregation is the major obstacle at higher protein concentrations for the commercial application of *in vitro* folding processes. However, renaturation at low protein concentrations gives large reaction volumes, resulting in higher costs. This problem can be solved by using the correct renaturation procedure (Jaenicke, 1990). If the actual concentration of denatured protein can be kept below a critical level where aggregation does not predominate, high yields of renaturation per volume of solution can be obtained (Goldberg *et al.*, 1991).

2.1.4 Current state of protein refolding

The relation between the amino acid sequence of a protein and its conformation came from the work of Anfinsen (1973), on ribonuclease. Ribonuclease is a single polypeptide chain consisting of 124 amino acid residues containing four disulphide bonds. Anfinsen found that when the fully reduced protein containing eight SH groups was allowed to reoxidize under denaturing conditions of 8M urea, a mixture of products was obtained containing many or all of the possible 105 isomeric disulphide bonded forms. The ribonuclease reoxidized in this way had only 1% of the enzymatic activity of the native protein. However, when the urea was removed and the "scrambled" protein exposed to a small amount of a sulfhydryl group-containing reagent such as mercaptoethanol, disulphide interchange took place and the mixture was converted into a homogeneous product which was indistinguishable from the native ribonuclease. These experiments clearly pointed out the fact that the information needed to specify the three-dimensional structure of ribonuclease was contained in its amino acid sequence.

To understand how a protein folds to its active conformation, one must know the pathway between the folded and unfolded states. A pathway can be defined by the intermediate states and the transition states occurring between the initial and the final states. The elementary model for a folding reaction is given by the two-state process shown below.

Denatured or unfolded **U↔N** Native or folded **(2.1)**

The reactions from right to left are both first-order steps and the ratio of the rate constants give the equilibrium constant for the reaction. Both thermal and denaturant-induced unfolding transitions are described by the two state model. Due to the complexity of some reactions this equation is not adequate to explain the large amount of experimental data. The rate of folding a protein is specific to

that protein and may take days to reach completion. The model below describes another model for folding.

$$\begin{array}{l} \mathbf{D \leftrightarrow\ I \leftrightarrow\ N} \\ \qquad\ \downarrow \\ \qquad\ \mathbf{A} \end{array} \qquad \textbf{(2.2)}$$

The model shows that from the denatured state the protein refolds to an intermediate state (or several intermediates) where D is the denatured protein and I is the intermediate form of the protein. The change to the intermediate state is usually a fast transition. The intermediate state shows some secondary structure since it is partially folded. From the intermediate state there are two possibilities for folding. The protein can refold into the native structure N but this step is slow and rate-limiting. The second possibility is for the intermediate to aggregate, A. The aggregation reaction is irreversible and of second order or higher whereas the refolding reaction is first order. Several models have been proposed for the pathways for folding and unfolding (Creighton, 1992).

2.1.4.1 Kinetic competition between refolding and aggregation

Ideally, renaturation procedures should be performed at high protein concentration. However, high protein concentrations increases the tendency for proteins to aggregate. A common observation is that the yield of renatured protein decreases as the concentration increases due to the aggregation of proteins (Zettlmeissl *et al.* 1979). Burton *et al.* (1989) reported that up to 94% refolding of human serum albumin (HSA) could be achieved using a relatively high protein concentration (5 mg/ml). They refolded the protein via dialysis which was found to be preferable to dilution as a means of chaotrope (urea) and reductant (2-mercaptoethanol) removal. However, in general, whenever renaturation is performed using high protein concentrations inactive aggregates are predominantly formed. It is assumed that the major cause of aggregation is the insolubility of the denatured state (Goldberg *et al.*, 1991).

When protein molecules are unfolded their hydrophobic cores are exposed to the solvent and aggregation results from non-specific interactions between hydrophobic regions of different polypeptide chains. London *et al.* (1974) have proposed that the precursors in aggregation reactions were partially folded intermediates populated during refolding and the association involved specific sites on these species. Goldberg and Zetina (1980) showed that these could be either segments of secondary structure that had not been packed against each other or larger domains that had not associated. When refolding is limited by the slow domain-pairing step then intermediates accumulate where at least some of the domains have already folded but have not interacted properly. This intermediate with Folded but Unpaired Domains, the FUD intermediate, leads to the kinetic competition between folding and aggregation (Creighton, 1992).

In the native protein each folded domain interacts with other parts of the chain by one or more specific sites which can give rise to two sets of different reactions, Creighton (1992):

1. Intramolecular interactions which are unimolecular and the rate of formation should not depend on concentration.
2. Intermolecular interactions which are multimolecular and the rate is expected to increase rapidly with the concentration.

At low protein concentrations aggregation should be slow and intramolecular interactions leading to the native state would be expected to occur over aggregation. At higher protein concentrations aggregation should be faster than the (first-order) formation of intrachain interactions resulting in a greatly reduced yield of renatured protein, Goldberg *et al.* (1991). Brems (1988) has shown a similar model to account for the concentration dependence of both the rate and the extent of renaturation during the refolding of bovine growth hormone (bGH). He described the refolding of bGH by a first order kinetic equation that contained two exponential terms. One of these exponential terms (corresponding to the slower

phase) was dependent on protein concentration while the other (corresponding to the faster phase) was not.

Mitraki and King (1989) and Schein (1990) have described aggregation as some partial folding occurring. The extent and rate of aggregation are greater in conditions that favour a mixture of folded, partially folded and unfolded states, than in strongly denaturing or strongly native conditions. Wang and Cleland (1991) have studied bovine carbonic anhydrase B (CAB) and have found that by using a co-solvent such as polyethylene glycol the rate of aggregation is decreased and the efficiency of folding increases. The co-solvent interacts with the hydrophobic portion of the polypeptide in CAB and inhibits the aggregation with other proteins.

2.1.4.2 Refolding processes

Many methods are available for refolding proteins from a denatured state. The methods used involve the reduction in concentration of the denaturing agents which are commonly GuHCl or urea. The techniques include dilution, dialysis and diafiltration (Vicik, 1990).

Dilution involves the addition of a buffer to a protein solution which reduces the denaturing reagent concentration. However, problems may be encountered using this technique on a large scale. Firstly, the downstream processing volumes are large which increases the purification costs. Secondly, rapid dilution with buffer can often give lower than expected levels of active protein due to the formation of protein aggregates (Goldberg *et al.*, 1991). London *et al.*, (1974) have refolded *E. coli* tryptophanase by dilution.

Dialysis is used to refold proteins by selective denaturing reagent removal. Dialysis is the principle by which separation of substances occurs via a semipermeable membrane. Small molecules and ions diffuse through the membrane readily whereas large molecules and colloidal particles pass through

slowly or not at all (Vicik, 1990). The refolding of proteins via dialysis can give biologically active proteins but since it is a diffusion limited process it is extremely slow. This is the main reason why dialysis is not used on a large scale production.

Diafiltration is the process whereby a buffer is continuously added and removed at an equivalent rate which keeps the protein in a constant volume environment. It is then filtered through a semipermeable membrane which selectively removes the denatured and other small molecules while keeping the protein of interest.

2.1.4.3 Factors influencing refolding rates and yields

The same factors influencing protein stability also influence the refolding yield of proteins these include pH, temperature, ionic strength and denaturing reagent concentration. Creighton (1978) suggests that protein refolding rates depend on the final environmental conditions. Environmental conditions influencing refolding rates include temperature, pH, ionic strength and denaturing reagent concentration. Kuwajima and Schmid (1984) have shown the refolding rates of lysozyme increase with increasing temperature. Epstein and Goldberger (1963) found that the rate of reactivation of reduced lysozyme increased with increasing pH of the reoxidation mixture. A three-fold increase in the rate of reactivation was obtained when the pH was raised from 7.5 to 8.0. The reactivation of lysozyme at 24°C and 34°C was studied and reactivation was found to be significantly faster at the higher temperature. However, there are other factors that have been observed to affect the yield of active protein during a refolding process and these will be briefly discussed as follows. The refolding process may be determined by the inactivation and aggregation rates of the protein. Temperature, pH, ionic strength and denaturation concentration also effect the aggregation rate. Other parameters are also important and these include protein concentration and the rate at which the denaturant is removed.

One of the most important parameters influencing aggregation rate is protein concentration. Goldberg *et al.* (1991) found that the recovery of denatured and reduced lysozyme was optimal at low protein concentrations (15 μg/ml). The decrease in yield at high concentrations was explained by the kinetic competition of refolding and aggregation.

Creighton (1978) observed that unfolded proteins were generally insoluble under poor denaturing conditions. Under these conditions the proteins tended to aggregate. The proteins include β-galactosidase, tryptophanase and elastase (Vicik and Clark, 1991). However, low concentrations of denaturant have been found to favour protein refolding. Goldberg and Orsini (1978) found that by incubating chymotrypsinogen at low denaturation concentrations refolding was promoted. The refolding of chymotrypsinogen from 6M guanidine hydrochloride was optimized by diluting to ~1.2M GuHCl in the presence of reduced and oxidized glutathione.

2.1.4.4 Examples of experimental observation of protein folding and unfolding

Most experimental studies of protein folding have been conducted *in vitro*. Before studying refolding it is necessary to unfold the native protein. This has been performed by changing the environmental conditions such as temperature, pressure or by adding a denaturant such as urea or GuHCl (Tanford, 1968, 1970).

With small globular proteins a single abrupt transition between the folded and unfolded state is generally observed irrespective of the parameter used for unfolding. As mentioned earlier, the equilibrium population of protein molecules may be approximated by a two-state model between fully folded (N) and unfolded (U) molecules:

$$U \leftrightarrow N \qquad (2.1)$$

Each molecule is either fully folded or fully unfolded, although small microscopic variations are possible within the two states (Tanford, 1978). Intermediates of partially folded molecules are generally thermodynamically less stable than the fully folded or the fully unfolded states. As conditions are changed the equilibrium transitions between U and N are very abrupt and therefore cooperative in nature.

However, folding reactions are not always as simple as the two-state process and many reactions are very complex. There are a number of explanations for the apparent complexity of folding transitions:

1. Two or more structural domains may be present in the native state each of which maintains its folded conformation independent of the other. Each of these domains could obey the two-state behaviour but this two-state behaviour of the entire protein would not be observed if the individual domains unfold under different conditions.

2. Under poor denaturing conditions unfolded proteins are generally insoluble and tend to aggregate.

Vincent *et al.* (1971) studied the unfolding of bovine pancreatic trypsin inhibitor (BPTI) with the three disulphide bonds kept intact and demonstrated the stability of its folded state under normal denaturing conditions. The native protein changes very little in extremes of pH, 6M GuHCl or 8M urea. However, when two or more denaturing conditions are used, full unfolding is observed and the protein behaves like a cross-linked random chain. The folding transition appears to be fully reversible unless the covalent structure has been modified, such as at high pH.

To understand the phenomenon of how proteins attain their folded state from equilibrium studies of the folding transitions observations are required to be made in the region of the transition mid-point under denaturing conditions often far from those considered normal (Tanford, 1978). The observations are then generally extrapolated to the conditions of interest. The denaturing effect is not

completely understood even for urea and GuHCl. The mode of action of denaturants has concentrated on preferential direct binding to the unfolded protein (Tanford, 1970) but little evidence has been found. It appears that denaturing agents act in an indirect manner by primarily disrupting the unique properties of water, which are ultimately responsible for the stability of the folded state.

There is a vast amount of published research on refolding processes, kinetics, folding pathways and factors that influence refolding rates and yields. One of the major obstacles in obtaining high yields of renatured proteins appears to be the formation of aggregates. Although the refolding processes of many proteins have been studied there has been no general protocol for large scale refolding. There is therefore a need for proteins to be refolded in large quantities in a short time and at higher concentrations than are currently available.

2.1.4.5 Techniques for monitoring conformational changes in proteins

The biological activity of a protein requires the correct three-dimesional structure to be in the native state. This is the simplest method used to characterise the state of the protein. The measurement of the specific enzymatic activity is the method of choice since it is fast, sensitive and highly specific. Apart from biological activity, more powerful methods used in folding studies are the spectral properties of proteins. The spectral properties of a protein depend upon its molecular environment and upon the mobility of its chromophores. Two methods, fluorescence and circular dichroism have been used for this project and are discussed below.

2.1.4.5.1 Fluorescence

Fluorescence may be observed when an excited electron returns from the frist excited state back to the ground state. This can be described as the loss of energy from an excited state molecule and results in the emission of light. Fluorescence is

a technique used for the investigation of conformational changes that may occur within a protein molecule. The fluorescence of proteins may be attributed to phenylalanine, tyrosine and tryptophan residues. For proteins in particular, tryptophan and tyrosine can be excited by high energy light (ultraviolet) and then return to the ground state of the electrons in the Π orbitals of aromatic rings which results in the emission of light in the visible range, fluorescence. Whenever proteins contain all three of these amino acids fluorescence is normally dominated by the contribution of the tryptophan residues because the absorbance values for tryptophan at the wavelength of excitation and quantum yield of emission are greater than the values of tyrosine and phenylalanine.

The intrinsic fluorescence of proteins can then be measured by exciting the protein with ultraviolet light and measuring the emission in the visible range. A typical excitation wavelength is 280 nm which excites both tyrosine and tryptophan residues within the protein. However, to avoid the additive fluorescence of both of these residues an excitation wavelength of 296 nm can be used to excite primarily the tryptophan residues since tyrosine molecules absorb less energy than tryptophans at this wavelength (Creighton, 1990). The emission spectra produced at both of these excitation wavelengths will yield insight into the conformation of the protein.

2.1.4.5.2 Circular Dichroism

Circular dichroism (CD) gives information about the unequal absorption of left- and right-handed circularly polarised light by optically active molecules. CD spectra of proteins result primarily from the spatial assymetry of the amino acids in the backbones of the macromolecules. CD signals may be observed in the same spectral regions where the absorption bands of a particular compound are found, provided that the respective chromophores or its molecular environment are asymmetric.

CD can be used as a means of following the denaturation and the refolding of denatured proteins. CD analysis rarely gives absolute information about structure but it is very sensitive to changes in protein conformation. If there is a change in the CD spectrum of a protein then a conformational change must have occurred. The denaturation of proteins is always accompanied by alteration of the CD spectrum which indicates the loss of α and β structure and the enhancement of the random-coil spectral components and also a loss in the tertiary structure. Hence, denaturation can be followed by plotting ellipticity at a particular wavelength as a function of the denaturing conditions. Molar ellipticity [Θ] (in degrees) can be calculated from the equation shown below:

$$\textbf{Molar ellipticity } [\Theta] = \frac{\Theta \times 100 \times Mr}{C \times d} \qquad \textbf{(2.3)}$$

where Θ is the measured ellipticity in degrees, C is the protein concentration in mg/ml, d is the path length in cm and Mr is the molecular weight of the protein. The forces that are responsible for determining the three-dimensional structure of a protein are also of great interest. These can be studied by examining helix-coil transitions. CD can add another dimension to the analysis if the amino acid sequence is known, since it is possible to look directly at the environment of aromatic amino acids and of disulphide bridges beacuse they have CD bands at characteristic wavelengths. Therefore, it is possible to detect changes in the neighborhood of particular amino acids caused by the destruction of hydrophobic forces or hydrogen bonds. The secondary structure of proteins are studied in the far-uv range (190-230 nm) but the protein needs to contain substantial proportions of α-helix and β-sheets (> 30%) for spectra to be observed. The near-uv region (250-300 nm) originates from the aromatic amino acids. These two spectral regions tell us different kinds of information about the structure of the protein.

CD bands from the amide region contain information about the peptide bonds and the secondary structure of proteins. Changes in the secondary structure of proteins can be monitored during the course of any structural transitions such as unfolding

of the molecule. The CD bands in the near-uv region may be observed when the aromatic side chains are immobilized in the folded protein and thus transferred to an assymetric environment. In the absence of any ordered structure the CD of aromatic residues is small. The near-uv CD spectrum of a protein can be used as a sensitive criterion for the native state. The spectra produced can be used as a fingerprint for the correctly folded conformation. CD is therefore a useful technique for monitoring structural transitions within proteins. CD differences between the native and the unfolded proteins are usually large in both spectral regions. Denatured proteins usually give an aromatic CD of zero.

2.1.4.6 Methods for improving refolding yields

Refolding proteins *in vitro* has been shown to be optimal at low protein concentrations. The decrease in yield which occurs at high protein concentrations can be related to the kinetic competition between refolding and aggregation. When unfolded protein molecules expose their hydrophobic patches then aggregation results from the nonspecific interactions of the hydrophobic regions of different polypeptide chains. In order to avoid the aggregation reaction the unfolded molecules need to bind these critical interactive sites in the unfolded state which would therefore prevent misfolding and/or aggregation. A few methods have been described below which are currently being investigated in this area.

Cleland (1986) has used polyethylene glycol (PEG) to improve the recovery of active bovine carbonic anhydrase B (CAB). During the refolding pathway of CAB an intermediate was formed which was observed to aggregate and form dimers and trimers prior to precipitation. PEG enhanced refolding by binding to a single site on the first intermediate in the refolding pathway of CAB. The application of PEG was further investigated by Cleland (1986) using three recombinant human proteins: deoxyribonuclease (rhDNAse), tissue plasminogen activator (rtPA) and gamma interferon (rIFN-γ). The refolding of rhDNAse and rtPA with PEG (3350 MW) prevented aggregation and increased the recovery and activity of the protein. Studies with PEG revealed that there was a stoichiometric relationship between

the polymer and the refolding protein. The polymer did not enhance the rate of refolding, but merely slowed down the competing aggregation reaction.

Carlson and Yarmush (1992) have used monoclonal antibodies (MAbs) to enhance the yield of S-Protein (a fragment of RNase A) during protein folding. The increase in the recovered enzymatic activity was directly related to the MAb concentration and it suggested that these antibodies assist refolding by direct interaction at specific parts of the protein.

Another more recent area is the role of chaperonins in protein folding. The concept of molecular chaperones originated from studies of the chloroplast enzyme rubisco (ribulose 1,5, bisphosphate carboxylase) which fixes carbon dioxide in plant photosynthesis (Ellis, 1990). The chaperonin proteins groEL and groES belong to a family of heat-shock molecular chaperones, found in prokaryotes and in eukaryote organelles (Goloubinoff *et al.*, 1991). Chaperonins meet the first criterion expected by their definition. Chaperonins cannot rescue the protein once aggregated but these moleculeas are capable of associating with proteins and preventing aggregation. Buchner *et al.* (1991b) studied the refolding of citrate synthase including the molecular chaperone GroE. *In vitro* denaturation of this protein is almost irreversible since it forms aggregated protein rapidly. It was found that the yields of reactivated protein were increased by the addition of GroE. GroE inhibited the aggregation reactions that compete with folding as shown by specific suppression of light scattering. The GroEL quickly forms a complex with the unfolded molecule and protects it from aggregation. Zardeneta and Horowitz (1994a) claim that the use of chaperones for refolding medically important recombinant proteins is not economically feasible due to the current high costs in producing them.

Recently Batas and Chaudhuri (1996) have used size-exclusion chromatography to refold lysozyme and bovine carbonic anhydrase (CAB) at high initial concentrations (80 mg/ml). Size-exclusion chromatography matrices are used to perform buffer exchange, remove aggregates and fold the proteins. The

aggregation reaction due to the nonpecific interactions of the partially folded molecules was reduced due to the slower diffusion of protein through the gel-filtration media. Lysozyme and CAB were recovered (63% and 56%) having specific activities of 104% and 81% respectively.

Detergents have been used to facilitate the reaction of GuHCl denatured rhodonase (Tandon and Horowitz, 1986 and Tandon and Horowitz, 1987). They used the non-ionic detergent lauryl maltoside at concentrations of 0.1-5 mg/ml. No renaturation occurred below 0.1 mg/ml but ability to renature increased cooperatively to concentrations of 5 mg/ml lauryl maltoside. This was due to the weak binding of the detergent to the intermediate rhodonase conformers which prevented aggregation. These results suggest that refolding of the denatured protein involves an intermediate with exposed hydrophobic surfaces which partition into active and inactive species and lauryl maltoside stabilises these surfaces which then prevents aggregation and promotes refolding.

Finally, the use of reversed micelles for refolding proteins is another interesting and challenging area. Reversed micelles as discussed earlier contain a small amount of water surrounded by a layer of surfactant dissolved in an organic medium. The unfolded protein molecule may be introduced into the micelle together with its chaotrope where it can be individually refolded without aggregating with other protein molecules. Conditions in these systems can be manipulated in order that only one unfolded protein molecule is transferred into an individual micelle. GuHCl denatured RNase has been successfully refolded in an AOT/isooctane reversed micelle but the protein was transferred in 1 M GuHCl (Hagen *et al.*, 1990). The problem with this process is that as the denaturant concentration is increased then the transfer of denatured protein decreases. This problem may be overcome by using micelles of a different nature (Garaza-Romos *et al.*, 1992). They showed 100% recovery of activity from GuHCl-denatured homodimeric enzyme triosephosphate isomerase when a CTAB/hexanol and n-octane/water reversed micellar system was used. The use of reversed micelles for refolding proteins is a relatively novel idea and requires further investigation.

In summary, from the vast literature regarding protein refolding, there still exists a major problem of aggregation for refolding proteins at high concentrations. The previous section showed advances that have been made recently in attempts to solve this major biotechnological problem, but there is still no unique method for refolding proteins. It has already been mentioned how reversed micelles may be used to solve the problem of aggregation. The next section contains a literature review with regards to reversed micelles and factors controlling solubilisation of proteins into reversed micelles.

2.2 Reversed micelles

Reversed micelles are aggregates of surfactant molecules with their polar heads facing inwards to the water and their hydrophobic tails dissolved in the continuous organic solvent. These nanometre sized droplets are generally spherical and produce phases which are thermodynamically stable and optically transparent. Their use in biotechnology simply arises from the fact that they are capable of solubilising water and hydrophillic molecules such as proteins and nucleic acids. Proteins have been shown to retain their conformation and activity inside the aqueous core of these micelles and the kinetic parameters are comparable to the aqueous environment (Luisi *et al.*, 1988).

The optical transparency of reversed micelles allows the use of standard aqueous spectroscopy techniques to characterise solutes together with the micelles. Laane *et al.* (1987) have reviewed these topics including various light scattering techniques and circular dichroism, fluorescence and NMR which can yield the size and shape of reversed micelles.

2.2.1 Formation

There are three methods that can be used to produce reversed micelles (Luisi, 1985). The first is to directly inject a small volume of a concentrated aqueous

solution into a large volume of surfactant in an organic solvent while mixing. The advantage of this technique is that the aqueous phase composition is known. However, the water content and solubilisation of proteins attained are low, particularly at high ionic strengths. The second method known as the phase transfer method involves mixing equal volumes of an aqueous phase with the surfactant-containing organic phase. In this method the distribution of the solutes reaches an equilibrium and protein can be transferred into the reversed micelles at higher ionic strengths than can be achieved by the injection method. Therefore, phase transfer can be used to transfer denatured proteins in GuHCl using this method. The other advantages of the phase transfer method are that it can reach much higher water contents and is straight forward to scale up for biotechnological industrial applications. However, the drawback of this method is that the water content and composition of the aqueous phase are unknown and have to be determined. The third method is the addition of a dry lyophilized protein to a reversed micellar solution. This method is applicable for proteins that are insoluble or only slightly soluble in an aqueous environment.

Matzke *et al.* (1992) have studied the mechanisms of protein solubilisation in reversed micelles using α-chymotrypsin and alcohol dehydrogenase (LADH) and found that the addition of a dry powder to a reversed micellar phase does not appreciably solubilise the protein until the diameter of the reversed micelle is similar to that of the protein. By using the injection method they found that protein solubilisation did not depend on micelle size whereas the phase transfer method allowed multiple protein occupancy since larger micelles were formed. To date in the literarture the phase transfer method is the most widely used and this method shall be used in the work reported.

2.2.2 General properties

Surfactant molecules are amphiphilic and consist of a non-polar region (normally a n-alkyl tail) and a polar region (a head group). There are four general groups which can be classified according to the nature of the head groups: anionic with a

hydrophilic group carrying a negative charge (eg. AOT), cationic with the hydrophile bearing a positive charge (eg. CTAB) nonionic, where the hydrophile has no charge (eg. Tween 85) and zwitterionic where the molecule contains, or can potentially contain, both a negative and a positive charge (eg. phosphatidylcholines). Surfactants prefer to be located at interfaces and tend to from an interfacial monolayer with the hydrophobic tails immersed into the organic phase and the polar head groups in the aqueous phase.

Reversed micelles have droplet diameters of 50 nm or less. Because these particles are much smaller than the wavelength of visible light they are normally transparent or slightly bluish due to Tyndall scattering. Reversed micelles may be formed both in the presence and the absence of solubilised water. The type of reversed micelles in this study contain water and are also known as microemulsions. Reversed micelles can be considered to be a dispersion of a small amount of water in a continuous oil phase. The surfactant molecules form spheroidal aggregates with the external shell made up from the hydrocarbon tails and the polar or charged head groups and any associated counterions being localized in the interior of the aggregate.

The composition of a reversed micelle is often defined in terms of the molar ratio of water to surfactant, Wo (or R), which can be defined as:

$$\mathbf{Wo = [H_2O] / [surfactant]} \qquad \mathbf{(2.4)}$$

The parameter Wo is particularly important as a means of adjusting the droplet size. Droplets of a chosen size may be prepared by controlling the Wo value. If all the surfactant molecules are located as a monolayer at the interface, the radius of the water core of the droplet, rw, may be calculated as shown below. Assuming a monodisperse size distribution of the micelles with water core radius, r, the total volume, V, of the water pools in 1 litre of solution is given by:

$$\mathbf{V = (4/3)\,\Pi\, r^3\, N_m} \qquad \mathbf{(2.5)}$$

where N_m is the number of reversed micelles per litre of the total system. If the overall molecular concentration of water in the system is given by $[H_20]$ then one has:

$$\mathbf{V = v_{H20} N_A [H_20]} \qquad \mathbf{(2.6)}$$

where N_A is the Avogadro number and v_{H20} is the volume of an individual water molecule. Similarly, the total surface area, A, of the reversed micelle water cores in 1 litre of a solution is given by:

$$\mathbf{A = 4 \Pi r^2 N_m} \qquad \mathbf{(2.7)}$$

If a_{SURF} is the area occupied by a surfactant head group at the oil/water interface and the assumption that all the surfactant is bound, then

$$\mathbf{A = a_{SURF} N_A [SURF]} \qquad \mathbf{(2.8)}$$

and dividing equation 2.5 by equation 2.6, and equation 2.7 by equation 2.8, we have:

$$\mathbf{r\ (water\ core\ radius) / nm = (3\ v_{H20} / a_{SURF}).\ Wo} \qquad \mathbf{(2.9)}$$

For an AOT stabilized water-in-oil microemulsion (reversed micelle) inserting the values of 0.03 nm^3 for the molecular volume of water and 0.6 nm^2 as the AOT area per head group at the interface equation (2.9) becomes :

$$\mathbf{r(water\ core\ radius) / nm = 0.18 \times Wo} \qquad \mathbf{(2.10)}$$

This treatment makes two assumptions:

1) There is a monodisperse distribution of water droplets and

2) The AOT-surfactant skin is able to take a wide variety of curvatures

allowing the radius to be continuously varied.

Equation 2.10 applies to a good approximation to AOT systems and has been suported by data produced from small angle neutron scattering, Toprakcioglu *et al.* (1984). If the surfactant is not loctated at the oil/water interface, then no simple relationship can be expected between r and Wo.

Surfactant geometry plays a crucial role in the determination of the type of micelle formed. Mitchell and Ninham (1981) have described the surfactant packing parameter :

$$v / a_h l \qquad (2.11)$$

where v is the volume of the hydrophobic tail, a_h is the head group area and l is the length of the fully extended tail. Generally single chain surfactants such as sodium dodecylsulphate (SDS) form normal micelles. In this case $v / a_h l < 1$ and oil in water microemulsions (o/w) are formed having positive interfacial curvature. However, in the opposite case when using di-chain surfactants such as AOT, $v / a_h l > 1$ water in oil microemulsions (w/o) or reversed micelles are formed having negative interfacial curvature. Di- and tri-chain surfactants tend to form reversed micelles since v is increased with little change in a_h or l so that a structure with negative curvature is favoured.

The sodium salt of the di-chained anionic surfactant sodium bis, 2-ethylhexylsulphosuccinate (Aerosol-OT) or Na(AOT) has been the most widely used surfactant for the formation of reversed micelles. AOT consists of two branched alkyl tails with a negatively charged SO_3^- head group, and a Na^+ counterion (Appendix 1). The alkyl tail and anionic head group unit is usually referred to as AOT, and the commercially available sodium salt is termed Na(AOT). Na(AOT)- stabilised microemulsions have been studied using many different structural and physical techniques and over a wide range of experimental conditions (e.g. chain length of continuous alkane Robinson *et al.* (1984) and

temperature and pressure Howe (1984)). AOT is one of the most studied surfactants in this area of research because it forms reversed micelles very easily in a variety of organic mediums, the droplets formed are monodispersed and it is cheap. Another reason for its popularity is that no cosurfactant is required which means that there is no uncertainty about its distribution in the system, Luisi *et al.* (1988).

The published data shows that a narrow range of surfactants are used with the phase transfer method of proteins. Lye (1993) has summarised the published data and found that AOT is the surfactant of choice due to its physiochemical properties and phase behaviour studies. However, there are now a wide range of surfactants that are commercially available on the market that should be exploited in reversed micellar research. The data shows that investigations in this area have also been restricted to a small range of proteins. This is primarily due to the fact that the use of AOT limits one to the size of the protein that can be partitioned into its aqueous core. The literature and research is limited to low molecular weight proteins (< 80-100 kDa) with relatively high isoelectric points (pI > 6-7).

An area that seems to be ignored when using AOT and other surfactant containing reversed micelles is when they are used for protein separation processes. The difficulty lies in removing the final traces of detergent which may be bound to the protein. This is important since most surfactants are toxic and tend to have denaturing effects on proteins. Therefore, the development of a reversed micellar system used for pharmaceutical proteins should contain naturally occurring surfactants rendering them safe to use as food additives. This area of research has been poorly investigated and there is scope for exploration.

2.2.3 Phase behaviour and the types of microemulsion

Since surfactants tend to be located at interfaces (air/water, water/oil, etc.) their ability to self-associate allows the formation of a wide range of organised assemblies. These include micelles, reversed micelles, microemulsions and liquid

crystals. A schematic triangular phase diagram for a water/surfactant/oil system is shown in Figure 2.2 clearly illustrating the organised assemblies and showing their stabilities. The single phase microemulsion is shown in the shaded region of the diagram which contains droplets as a the result of molecular self-assembly of the system (Robinson, 1991). Each side of the triangle represents a binary mixture of two components along that side. The composition of any point in the triangle may be obtained by drawing three lines from the point parallel to each side. The mole fraction (volume fraction and weight fraction may also be used) of the components is represented by the distance along these lines.

The type of oil used for the formation of reversed micelles can influence protein solubilisation by changing the size of the micelles and the nature of the interface (Mall, 1992). Long chain oils can penetrate the surfactant layer to a greater extent than the shorter chain oils resulting in smaller aqueous cores. Hexane micelles are larger than dodecane micelles and should be able to solubilise more protein.

The phase diagram shown in Figure 2.2 does not take into account the presence of salt and proteins. There has been no study on the influence of proteins on the phase diagram to date.

There are basically three types of microemulsion which have been identified, the formation of which will depend primarily on the relative amounts of water and oil in the system. Oil-in-water microemulsions are considered to have a small amount of oil dispersed in a continuous water phase (also known as L_1 phase). In this system the surfactant molecules adopt a positive curvature, as for a micelle, with the polar head groups directed into the water and the non-polar tails pointing inwards. The oil is present in the interior of the droplet.

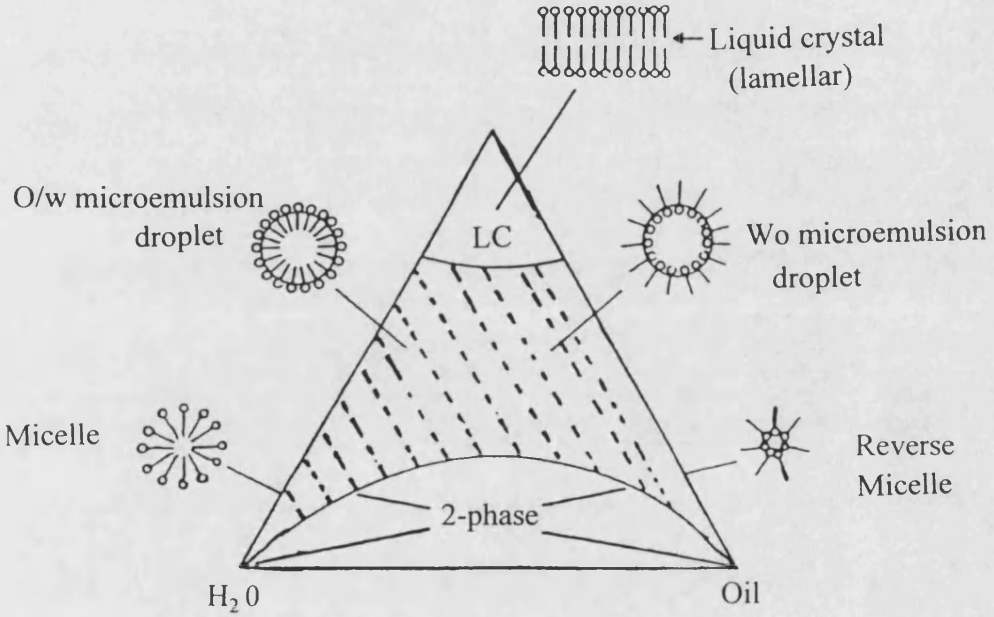

Figure 2.2 Schematic triangular phase diagram (shaded region shows microemulsion), adapted from Robinson (1991).

Water-in-oil microemulsions(w/o) are similarly considered as a dispersion of a small amount of water in a continuous oil phase (also known as an L_2 phase). The surfactant molecules form spherical aggregates, but in this case the external shell is made up from the hydrocarbon tails with the polar or charged head groups and any counterions located in the interior of the aggregate. In these conditions the surfactant exhibits negative curvature and water is solubilised in the core of the microemulsion droplet. A third type of microemulsion can be formed under certain conditions but mostly when volumes of oil, surfactant and water are similar. These bicontinuous systems are frequently present and exhibit fluctuating curvature (both positive and negative), Robinson (1990).

In the surfactant rich corner of the phase diagram liquid crystal structures are formed. Where the surfactant is insufficient ie. near the water/oil binary mixture line, the mixture separates out into two phases.

2.2.4 Protein partitioning into reversed micelles

Section 2.1.1 describes how proteins can be partitioned into reversed micelles using three different techniques. The actual mechanism for the partitioning of proteins into reversed micelles is not well understood but if two phases, a protein containing aqueous phase and a reversed micellar organic phase, are mixed together materials may be exchanged across the interface. If the organic solvent has neglible miscibility with the water and the surfactant has negligible solubility then there exists the possibility for water, protein and other solutes to transfer. These materials can partition across the interface until an equilibrium is achieved or the phases are separated.

Since hydrophillic proteins are not soluble in organic solvents it is unlikely that these proteins can enter the micellar core by diffusion through the organic phase after transferring across the interface. The protein must diffuse towards the aqueous/organic interface, which consists of a monolayer of surfactant molecules, form a micelle around itself carrying both water and surfactant into the organic

phase, Göklen (1987). Proteins approach this interface at different speeds and only a fraction of these collisions result in the solubilisation process. For the reverse process, a protein filled micelle must collide with the interface and the protein and water readsorb in the aqueous phase whilst the surfactant returns to the interface.

2.2.4.1 Factors affecting protein transfer

There is a large body of evidence indicating that electrostatic interactions play a crucial role in the distribution of protein between an aqueous phase and an organic phase. This can be demonstrated by the effect of aqueous phase pH, ionic strength and the type and concentration of surfactant employed. The factors that can influence the partitioning of proteins into reversed micelles using the phase transfer process are described below.

2.2.4.1.1 The effect of pH

The partitioning of proteins into reversed micelles is related to the electrostatic interactions between the charges on the surface on the protein molecule and the surfactant head groups which form the surface of the reversed micelle. There is an attractive electrostatic interaction between a protein molecule and the surfactant head groups when the overall charge of the protein is opposite the charge of the surfactant head groups. The net charge on a protein may be determined by the different amino acid residues present on the surface. Since the surface contains both positive and negative charges there is a point when the protein has no surface charge ie. the balance between both charges is equal and this is known as the isoelectric point (pI). This may be determined by the ionisation of the exposed amino acid residues, ie. their individual pK_as, which is determined by the aqueous phase pH.

For anionic surfactants it may be hypothesised that the solubilisation of proteins into reversed micelles is favoured when pH values are below the pI of the protein while the opposite is true for cationic surfactants. Göklen and Hatton (1987) found

that the proteins ribonuclease A, cytochrome c and lysozyme could be completely solubilized using AOT reversed micelles and the solubilization took place in a range of 4-6 pH units just below the pI's of the three proteins. The decline in the amount of lysozyme and cytochrome-c solubilised at low pH < 7 was due to protein denaturation and the formation of a precipitate at the interface. Marcozzi *et al.* (1991) studied the effect of pH on the transfer efficiency of α-chymotrypsin with a number of different salts (NaCl, KCl, LiCl and $CaCl_2$) into AOT/isooctane micelles. All the salts behaved similarly with a maximal transfer efficiency around pH 5 which was just below the pI (8.5) of the protein. Matzke *et al.* (1992) compared the solubilisation of α-chymotrypsin into micelles using AOT compared to Aliquat-336, a cationic surfactant as a function of pH. When a positively charged surfactant is used, there should be a favourable protein-surfactant interaction above the pI of the protein and an unfavourable interaction below the pI. They found that for AOT, protein transfer increased and improved until the pI of α-chymotrypsin was reached then a decline in transfer was oberved. For Aliquat-336, only a small amount of protein was transferred at low pH (2-6) but transfer gradually increased as the pH of the aqueous phase passed through the isoelectric point.

At this point, it is important to note that the pH in the aqueous phase (pH_{st}), before solubilisation takes place is not necessarily the same as the pH inside the micellar water-pool (pH_{wp}). The properties of solubilised water are different to those of normal bulk water and will have a high charge density due to the ionisation of the AOT head groups, Smith and Luisi (1980). The pH_{wp} will therefore be different from pH_{st} , especially at Wo<10. At Wo>10, water in the core of the reversed micelles will exhibit properties similar to those of normal bulk water. Since solubilisation of lysozyme into AOT/isooctane reversed micelles is very pH dependent it becomes important to determine pH_{wp}. Smith and Luisi (1980) argued that the micellar water is a completely new solvent and measurements using an electropotential glass electrode (Menger and Yamada, 1979) or indicator dyes (Nome *et al.*, 1976) will have errors, as the techniques will not be calibrated for this new solvent. The problems associated with indicator dyes include the dye

used, the type of buffer, the Wo value and the surfactant used which relate to changes in their pK_a values. Another method to determine pH_{wp} uses ^{31}P chemical shifts of different types of phosphate buffers, at various pH values as measured in bulk water with the shifts as obtained in reversed micelles. For AOT reversed micelles this technique showed that the pH_{wp} was less than pH_{st} by between 0-1 unit. A new method in determining pH_{wp} is an enzymatic method using hydrogenase as a redox catalyst, Dijk *et al.* (1992). It was found that for AOT reversed micelles:

$$\mathbf{pH_{wp} = 0.66\ pH_{st} + 2.32} \qquad \mathbf{(2.12)}$$

and that pH_{wp} was independent of Wo. Figure 2.3 shows the pH of the water pool as a function of aqueous phase pH according to Equation 2.12.

The effect of pH on the partitioning of proteins of differing molecular weights may be explained by the fact that for larger proteins the size of the reversed micelle containing the protein must be larger than the empty micelle. This unfavourable transition must be compensated by more electrostatic interactions to make the solubilisation process feasible (Dekker 1990). By increasing the charge density of the protein by simple manipulation of the pH this may be overcome. Wolbert *et al.* (1989) have shown a linear correlation between the molecular weight of the protein and the difference between the pH of solubilisation and the pI of the protein and this correlation held for anionic and cationic surfactants. Wolbert *et al.* (1989) studied the uptake profile of 19 proteins as a function of the pH of the aqueous solution. They used an AOT/isooctane system and a cationic system consisting of trioctylmethylammonium chloride and nonylphenolpentaethoxylate (Rewopal HV 5) as the surfactant and octanol as the cosurfactant in an isooctane continuous phase. The pH where maximum solubilisation takes place has been described by the following equation:

$$\mathbf{pH_{optimum} - pI = 0.11 * 10^{-3}\ M_r - 0.97} \qquad \mathbf{(2.13)}$$

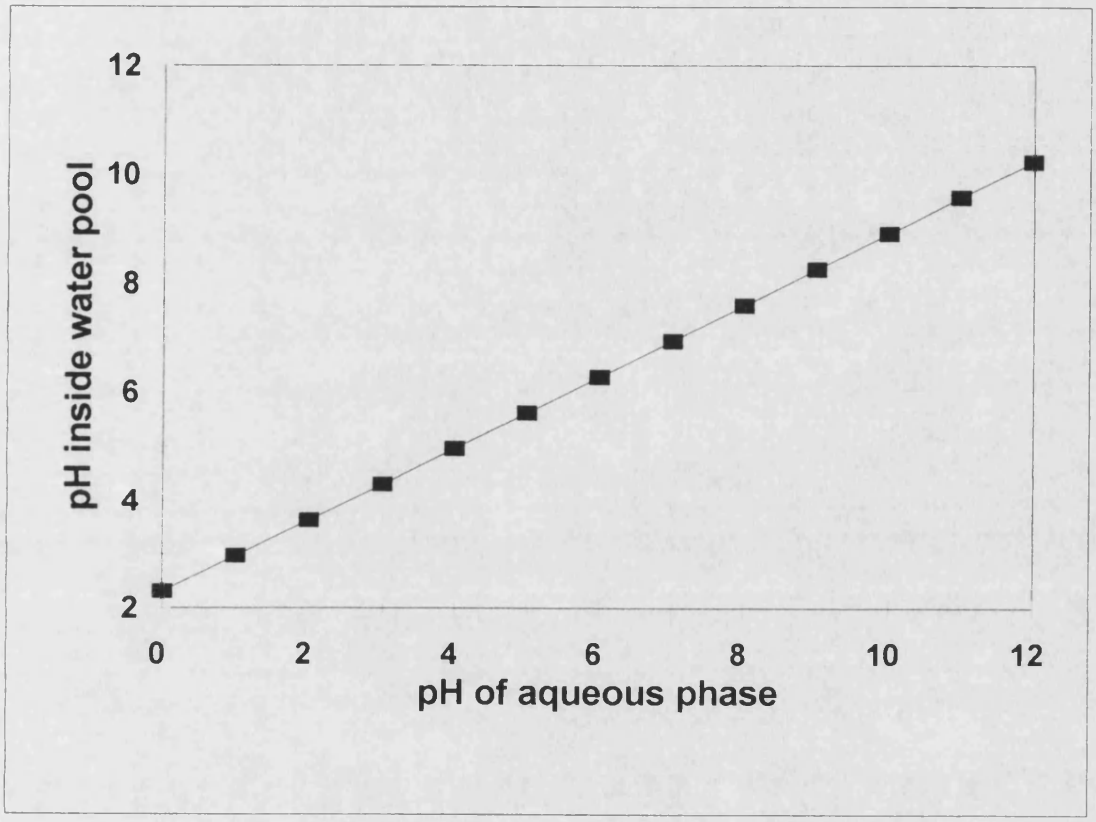

Figure 2.3. Theoretical plot of the pH of the water pool inside reversed micelles

The above data has been plotted from the work of Dijk et al. (1992) and shows pH values of the aqueous phase in comparison to the water pool of the reversed micellar phase. The data fits the equation $pH_{wp} = 0.66 * pH_{st} + 2.32$.

For the cationic micellar system, solubilisation of the proteins was only found at pH values above the pI where the proteins were negatively charged. Lysozyme was one of the proteins that did not transfer into the organic phase. A similar equation has also been derived for solubilisation of proteins in AOT/isooctane reversed micelles and has been described by the following equation:

$$\mathbf{pH_{optimum} - pI = 0.12 * 10^{-3} M_r - 1.07} \qquad \mathbf{(2.14)}$$

and by plotting $pH_{optimum}$ - pI vs M_r, it is observed that $pH_{optimum}$ - pI increases with M_r. Table 2.1 displays the literature data for the solubilisation of proteins in 50 mM AOT/isooctane reversed micelles obtained by Wolbert *et al.* (1989). The results showed that seven out of the ten data points could be fitted to the line: $pH_{optimum} - pI = 0.12 * 10^{-3} M_r - 1.07$. The conclusion for both anionic and cationic surfactants is that the size of the protein determines the charge density required for its transfer and thereby the pH of uptake. It becomes clear that charge interactions between the surfactant and protein are important but no relationship can be established between the percentage transfer into micelles and the number of charged groups in the proteins. A possible explanation could be that the charge density is not distributed homogeneously over the surface of the protein and can differ between proteins, (Wolbert *et al.*, 1989).

2.2.4.1.2 The effect of ionic strength

The ionic strength determines the amount of electrostatic shielding imposed on a charged surface. This phenomenon causes at least two important effects in reversed micellar extraction. Firstly it decreases the electrostatic interaction between the charged protein molecule and the charged interface in the reversed micelle. Secondly, there is a reduction in electrostatic repulsion between surfactant head groups which reduces the size of the reversed micelles, particularly at higher ionic strengths. Göklen and Hatton (1987) have shown differences in the distribution behaviour of lysozyme, cytochrome c and ribonuclease A in an AOT/isooctane reversed micelle as a function of KCl.

Table 2.1. Data on the solubilisation of proteins in 50 mM AOT/isooctane reverse micelles (reproduced from Wolbert *et al.* 1989).

Protein	Source	$M_r \times 10^3$	pH_{opt}	Transfe r	pI	pH_{opt} - pI
α-Amylase	Bacillus	55	5	15	5.3	0.3
BSA	Bacillus	68		0	4.9	
α-Chymotrypsin	bovine pancreas	25	6	100	8.5	2.5
α-Chymotryp -sinogen	bovine pancreas	25	7.8	100	8.8	1.0
Cytochrome c	horse heart	12.5	9.6	100	9.9	0.3
Elastase	porcine pancreas	26.5	7	65	8.5	1.5
Lysozyme	chicken egg white	12	10.8	100	10.9	0.1
Rennin	calf stomach	35	3.9	50	5.2	1.3
Ribonuclease	bovine pancreas	13.5	7	100	8	1.0
Trypsin	bovine pancreas	25	6.7	100	10	3.3
Trypsinogen	bovine pancreas	26	7.3	100	9.3	2.0

pH_{opt} is determined from data obtained by Wolbert *et al.* (1989). The isoelectric points (pI) were determined by Wolbert *et al.* (1989).

An increase in salt concentration resulted in a decrease in transfer for all the proteins but the concentration required to initiate the decrease was different for each protein and no rules could be deduced from this data.

Since reversed micelles are held apart by head group repulsions between like charges, oppositely charged ions introduced in the centre will screen these repulsive charges and shrink the micelles. If enough charges are introduced then the size of the micelle becomes smaller than the protein and no transfer is achieved. Therefore, low ionic strengths and pH < pI can be used to solubilise proteins with anionic surfactants and high ionic strengths and a pH > pI can be used for the backward transfer process (Fletcher and Parrot, 1988).

As the protein's charge depends on the aqueous phase pH and high ionic strengths can screen electrostatic interactions, it seem likely that an electrostatic interaction between a protein and the double layer will play a major role in protein solubilisation. If we consider a protein as a sphere of charge, with its net charge giving it an apparent charge density, there will be an electrostatic potential in the aqueous medium surrounding the protein. This potential may be described by the Debye-Huckel approximation (Atkins, 1989). According to this model, the electrostatic potential in an aqueous electrolyte adjacent to a spherical surface of charge decreases exponentially with distance from the surface:

$$K = \sqrt{\frac{8\pi e N_a I}{1000 \varepsilon k T}} \qquad \textbf{(2.15)}$$

where K^{-1} is the debye length (Å), N_a is Avogadro's number, e is the electronic charge, I is the ionic strength, ε is the dielectric constant of the medium and k is the Boltzmann constant and T is the absolute temperature. The debye length is a characteristic length for the range of the electrostatic potential and is proportional to $I^{1/2}$. I is given by:

$$\mathbf{I = 1/2\ C_i Z_i^2} \qquad \textbf{(2.16)}$$

where Z is the charge of each ion and C_i is the molar concentration. Increases in ionic strength of the medium will compress the double layer such that the electrostatic potential decreases more rapidly with increasing distance from the surface of charge. Leodidis and Hatton (1989) have presented work with the selective solubilisation of cations into AOT/isooctane reversed micelles. Their model distinguishes between different cations via their charge and hydrated size. Water uptake (Wo) into reversed micelles was found to decrease significantly as the salt concentration was increased and the actual magnitude of Wo at a given salt concentration depends on the type of salt used.

Dekker (1990) has shown the effect of pH on the transfer of α-amylase to a reversed micellar phase consisting of TOMAC in isooctane at different ionic strengths. He observed that as the salt concentration was increased a higher pH was required for transfer to occur, due to the protein having a higher charge density. Once again electrostatic interactions appear to be the dominating factor in the phase transfer method for protein extraction to occur. Dekker (1990) has shown that as the salt (NaCl) concentration increases, a higher pH (due to a higher charge density on the protein) was required for maximal phase transfer of α-amylase into reversed micelles consisting of TOMAC in isooctane. This confirms that electrostatic interactions are important in the phase transfer mechanism. Marcozzi *et al.* (1991) have studied the factors that affect the forward and backward transfer of α-chymotrypsin into AOT/isooctane micelles. They used four types of salts (KCl, NaCl, LiCl and $CaCl_2$) and found that protein was transferred at the lowest ionic strengths with KCl followed by $CaCl_2$, NaCl and LiCl. The size of these ions obviously played a part in the transfer of the protein. Abbot and Hatton (1988) have stated that ionic strength effects play a crucial role in the solubilisation process. As well as screening electrostatic interactions between the protein and surfactant head groups and forming smaller micelles another effect of ionic strength is to salt out the protein from the micellar water pool. This is due to the ionic species migrating to the micellar water pools and displacing the protein. Also, specific and non-specific salt interactions with the

protein or surfactant may modify the solubilisation behaviour and higher salt concentrations lead to more pronounced effects.

Andrews *et al.* (1994) has shown the effect of increasing the ionic strength of three salts $MgCl_2$, NaCl and KCl on the solubilisation of ribonuclease in 50 mM AOT/isooctane micelles. They found that $MgCl_2$ allowed protein transfer to occur over ionic strengths from 0.3 to 2.7 M which was significantly higher than the values for KCl and NaCl. The Mg^{2+} ion has an atomic radius of 0.66 Å. This is smaller than the K^+ and Na^+ ions and consequently shows that smaller ions do not screen the charges between the surfactant head groups as much as the larger ions thus allowing more protein to be transferred.

Marcozzi *et al.* (1991) are the only group that have shown that the amount of protein back extracted depends on the type and concentration of salt used in the forward transfer.

2.2.4.1.3 The effect of surfactant

As already discussed the distribution of the protein is dependent primarily on the difference in charge between the protein and charged surfactant head groups. The charge on the surfactant head group influences the pH range for protein solubilisation. Anionic surfactants such as AOT require pH < pI for efficient transfer and the opposite is true for cationic surfactants such as CTAB. AOT has been the choice of surfactant to date and thus there is a limit to the range of proteins (< 100 kDa) which can be studied. Given the number of surfactants commercially available it is interesting that AOT is the main surfactant used.

Woll *et al.* (1987) have shown that at a higher surfactant concentration there is a greater degree of protein solubilisation for a given pH. They found by increasing surfactant (AOT) concentration for the partitioning of RNase that the solubilisation curves became steeper as the pI of the protein was approached and the region where only partial transfer occurred became narrower.

Woll and Hatton (1989) and Göklen (1986) have shown that increases in the surfactant or cosurfactant concentration increases the amount of protein that can be solubilised in reversed micelles. Increasing surfactant concentrations generally results in larger spherical aggregates as shown by Göklen (1986) with Karl-Fischer titration and dynamic light scattering. At 0.1 M KCl the change in micelle size was slight between 25-75 mM AOT but increased more significantly at concentrations higher than 75 mM. Fletcher and Parrot (1988) have shown that there is a minimum concentration of AOT required for the solubilisation of lysozyme and this is the point at which the surfactant achieves monolayer coverage of the protein.

The use of non-ionic surfactants has received much less attention for protein solubilisation proceses. Microemulsions formed using non-ionic surfactants can include more water than the water of hydration of the surfactant making the droplet sizes larger, reaching 150-300 Å in diameter (Madhavan *et al.*, 1994). Therefore, larger proteins could be extracted into the cores of these micelles and not be size excluded as in the case with ionic surfactants. Madhavan *et al.* (1994) have used a sorbitan ester non-ionic microemulsion and extracted cytochrome c. They found that protein partitioning in these microemulsions is not a function of water content, alkyl chain length or polyethylene oxide molecular weight. It appears that the sorbitan group plays an important role in extraction, possibly by a weak protein-surfactant electrostatic interaction. There has been little work in the area of non-ionic microemulsions for protein separation processess. A possible advantage for using this type of microemulsions is that since the head groups posess no charge, the protein and surfactant may not bind together and separation would be simple. The disadvantage of using these systems is that a cosurfactant is required to form the microemulsions which introduces another component making a more complex system. Also non-ionic surfactants tend to produce polydispersed micelles.

Dekker *et al.* (1990) showed that the addition of a non-ionic surfactant (Rewopal HV5) to a TOMAC/isooctane reversed micellar system improved the partitioning of α-amylase and was a strong function of the ratio of non-ionic to ionic surfactant. Increases in the non-ionic component increased the transfer into the reversed micellar phase and took place over a wider pH range. The use of mixed micelles has also not received much attention and is another area of research that can be exploited.

2.2.4.1.4 Other factors influencing partitioning

Oils can influence protein partioning into reversed micelles by changing the size of reversed micelles formed (Jolivalt *et al.*, 1990a). Mall (1992) found that shorter chain oils such as hexane form larger micelles since they cannot penetrate the surfactant layer as much as the longer chain oils such as hexadecane.

Temperature is another parameter which may be used to influence the partioning of proteins into reversed micelles. Luisi *et al.* (1979) showed that by increasing the temperature from 25°C to 40°C, protein transfer was increased from 50 to 100%. Hagen (1989) showed that RNase transfer into micelles could be increased two fold by increasing the temperature form 10°C to 40°C. Increasing temperature does seem to improve the transfer process, but at higher temperatures the micelles become more unstable (Luisi, 1985) and several centrifugation steps are required in order to obtain a clear phase.

The phase volume ratio ($V_r = V_{aq} / V_{rm}$) is also found to affect protein transfer. Göklen and Hatton (1985) showed that 100% cytochrome-c could be extracted into 50 mM AOT/isooctane reversed micelles at V_r=1 and V_r=2 yielding a two fold concentration of the protein. Lye (1993) used V_r=5 and found that the maximum lysozyme concentration in the reversed micellar phase was 13.3 mg/ml.

2.2.4.1.5 The backward transfer process

The backward transfer process of proteins from a reversed micellar phase is not well documented as in the case of the forward transfer mechanism. Most of the studies so far are in the literature are based on the electrostatic interaction between the protein and surfactant with manipulations of pH and ionic strength. When using anionic surfactants proteins may be recoverd by increasing the pH since the negatively charged proteins repell the surfactant head groups. In addition, backward transfer of proteins from reversed micelles may also be achieved by increasing the ionic strength which screens interactions between the surfactant head groups thus shrinking the micelle. Reversed micelle extraction processes for large-scale continuous purification of proteins rely on methods to recover the proteins from reversed micelles. Some alternative methods that have been used are described below.

Lesser *et al.* (1992) have used an alternative backward transfer procedure which uses silica. Silica is added to the protein-containing reversed micelle and this causes the water to be stripped out from the AOT/isooctane micelles and the protein molecules to absorb to the silica particles. The recovery of the proteins from the silica into an aqueous solution is more efficient at alkaline pH (pH 8.0). The reason for this was as the pH rose upto the isoelectric points of the proteins α-chymotrypsin and trypsin (9.0 and 11.0), the electrostatic intercactions between the positively charged proteins and the acid Si-OH groups on the silica surface gave the silica particles a net negative charge under alkaline conditions. This decreased the binding tendancy of the proteins to silica as the pH was increased and approached the isoelectric point. They managed to recover 60-80% of the total protein (α-chymotrypsin or trypsin) retaining 80-100% specific activity at the end of the whole cycle. One of the drawbacks of this system was that the surfactant also adsorbed to the silica in quite significant amounts but no further investigations were made.

A new method based on isopropyl alcohol addition has been developed by Carlson and Nagaragan (1992). They studied the backtransfer of two proteins, porcine pepsin and bovine chymosin, and after complete solubilisation into AOT/isooctane micelles found that they could not back-transfer the proteins using pH values above their isoelectric points. However, they found the addition of 10-15% isopropyl alcohol to the aqueous phase resulted in complete back transfer of porcine pepsin and 70% of bovine chymosin was recovered. The recovered pepsin retained all of its activity under these conditions. This technique was tried for the backward transfer of lysozyme using 5-50% isopropyl alcohol but no protein was recovered in the resulting aqueous phase. Thus, the procedure is protein specific and no general rules can be made.

Gupta *et al.* (1994) have recovered a number of proteins and amino acids by dehydrating reversed micelles using molecular sieves. The products precipitate in the form of a solid powder which is relatively free of surfactant compared to the silica gel method. Almost complete precipitation has been demonstrated for α-chymotrypsin, cytochrome-c and tryptophan from AOT/isooctane micelles. This method of back extraction is different from others in that there are no requirements for manipulations of pH, ionic strength, temperature, pressure or solvent composition. The advantages of this technique over the others is that the hydrophillic components are recovered as a concentrated powder from the reversed micelles and there is no need for a crystallisation step. This dehydration is very general and could be used for any type of solute over wide ranges of pH, ionic strength and temperature. However, the authors did not determine the protein activity after recovery and this is one of the most important parameters.

Woll *et al.* (1987) found that the proteins RNase and concanavalin A could not be recovered from 50 mM AOT/isooctane micelles by using pH and ionic strength variations indicating that there are strong protein-surfactant interactions. However, they observed that addition of a small quantity of a more polar, water immiscible solvent to the reversed micellar system caused disruption of the micelles and both proteins were completely recovered. They found that addition of 10-20% by

volume of ethyl acetate to the AOT/isooctane solution resulted in recovery of both RNase and concanavalin A without any loss in activity. The main drawback for using this backward transfer method is that the solvent is no longer reusable for another extraction step since its solubilising power would be reduced considerably by the addition of the polar organic.

Dekker (1989) has used the effect of temperature to back extract α-amylase from a nonionic reversed micellar system. He found increasing the temperature of the micellar phase after forward extraction of the enzyme caused a separate aqueous phase to be formed containing the enzyme in a concentrated form. The excess aqueous phase was easily separated by centrifugation. This whole extraction procedure was performed in a continuous process and 73% of the enzyme activity was recovered.

2.2.5 Partitioning of denatured protein

The partitioning of denatured protein into reversed micelles has received little attention to date. The work by Hagen (1989) is the only example of denatured RNase being partitioned into AOT/isooctane reversed micelles using the phase transfer method. Very little is known about the mechanism of transfer of denatured proteins into micelles. Hagen (1989) showed that there was a significant difference in the amounts of native and denatured protein transferred into reversed micelles.

Hagen (1989) has observed that denatured RNase could be transferred into micelles under conditions that native RNase could not. The native form of the protein was fully folded whereas the denatured form has an unfolded, random coil structure. This enables the denatured protein to bind the positively charged guanidinium ions then the protein plus denaturant would be attracted to the negatively charged surfactant head groups. The increased interaction between the protein plus denaturant and surfactant may facilitate the denatured protein into the reversed micelles more easily than the native protein.

The other pioneering research in this area has come form the work of Tandon and Horowitz (1986, 1987) and Zardeneta and Horowitz (1992, 1994b). They have concentrated on refolding the enzyme rhodonase with the non-ionic detergent dodecyl-β-maltoside (lauryl maltoside). Insoluble aggregates were found to be formed when rhodonase, denatured in 6 M GuHCl was diluted in the aqueous environment for renaturation at protein concentrations > 1 μg/ml. The aggregation is due to exposure of hydrophobic surfaces that would become interdomain contacts of the correctly folded protein. Tandon and Horowitz (1986) have shown that refolding of GuHCl-denatured rhodonase involves an intermediate with exposed hydrophobic surfaces that can form active and inactive species. Lauryl maltoside can stabilise these surfaces thus preventing aggregation and yielding active protein.

In summary, it appears that there may be potential to exploit reversed micelles for refolding proteins. The protein could be partitioned into these micelles due to the selectivity of the electrostatic interactions involved and prevent the problem of aggregation encountered in biotechnology. However, very little is known about partitioning denatured protein molecules into reversed micelles. The fact that high protein concentrations (mg/ml) can be transferred into micelles, has implications that refolding may also be performed at higher concentrations than is currently achieved (μg/ml).

2.3 The overall process and aims of the project

A proposed method for refolding aggregated proteins is to use reversed micelles. Reversed micelles are aqueous phase droplets stabilized by a surfactant and suspended in an alkane solvent. Reversed micelles can offer an environment in which proteins may refold in isolation from one another thus reducing the probability of aggregation during refolding (Figure 2.4). The advantage of using such a system is that proteins may be partitioned into the aqueous core and

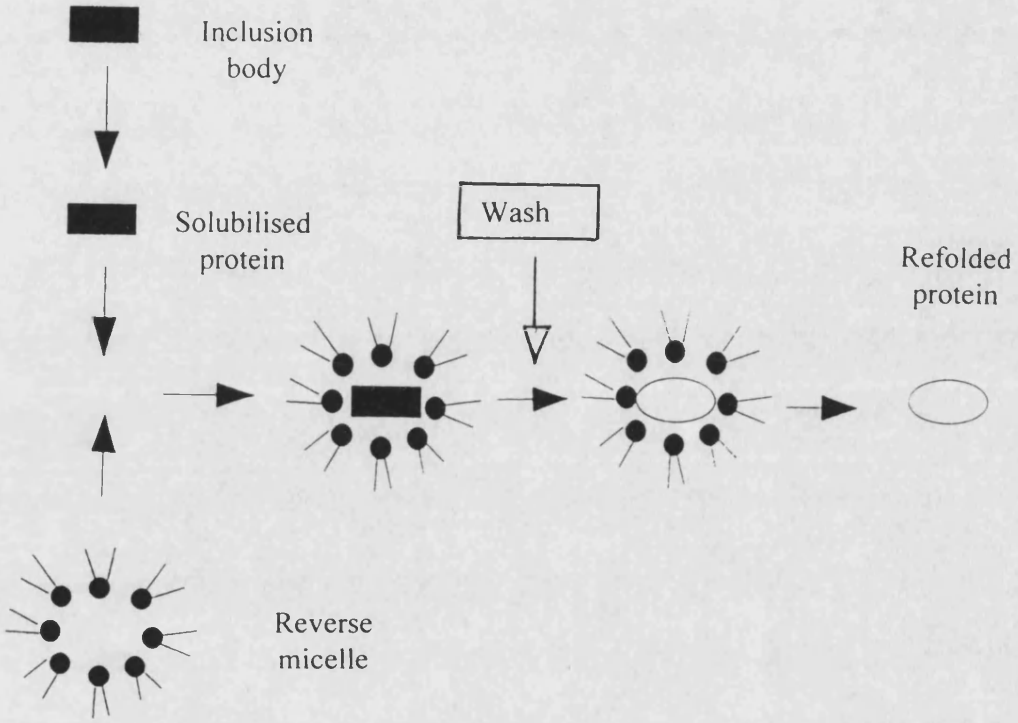

Figure 2.4 Schematic diagram for refolding proteins inside reversed micelles

conditions can be manipulated so that a maximum of one protein is transferred into one micelle.

The objectives of this research were as follows:

1. To establish the conditions for complete denaturation of the protein molecule.

2. To transfer denatured lysozyme into reversed micelles. This was the key step in the overall process since each unfolded protein molecule must be isolated into a micellar core of a reversed micelle to prevent aggregation.

3. To allow the protein to refold to its native conformation by removal of the denaturant inside the micelles. This involved a series of contacting stages with other reversed micelles containing the refolding buffer. The denaturant concentration was diluted to as low a concentration as was possible inside the micelles which retained the protein.

4. To provide the correct environment for formation of the native disulphide bonds allowing the protein to regain its native three dimensional structure, the reduction of denaturant is followed by addition of reduced and oxidised thiols when the protein contains disulphides.

5. The final step was to back extract the protein from the reversed micellar solution into an aqueous solution under conditions giving optimum transfer while retaining all the activity.

Each of the steps above needs to be considered individually and optimized in order for the overall process to be successful. The important steps were to chose the correct conditions which allowed partitioning of the protein into the reversed micellar system and to back extract the protein from the micellar solution.

To test the feasibility of this overall process it was important to select proteins that could be partitioned into reversed micelles. Lysozyme was chosen as the model for this project. An important reason for chosing lysozyme was that it is relatively small and can be partitioned into reversed micelles. It has been studied extensively in aqueous solutions and its individual refolding behaviour has been well-characterised. The protein has been selected on the basis that there is a wealth of knowledge in the literature and that the denaturation and renaturation procedures have been established.

2.3.1 Choice of system

The reversed micellar system chosen is another important parameter to be considered. There are now many surfactants that have been well-characterised and are known to form reversed micelles. The most common and widely used surfactant is Aerosol OT (di-2 ethylhexyl sulfosuccinate). It has the advantage of being cheap and forming reversed micelles under a wide variety of conditions. Reversed micelles are easily formed with the AOT/isooctane system giving a clear, thermodynamically stable solution. This enables the proteins to be studied in these systems using a wide variety of analytical techniques. There are a number of ways that reversed micelles can be prepared and these have been described in the literature review. The method chosen was the phase transfer method which involves mixing equal volumes of an organic phase with an aqueous phase followed by separation with centrifugation. This method allows the formation of reversed micelles in the presence of proteins and salts such as GuHCl.

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

Hen egg-white lysozyme EC 3.2.1.17 (52000 units/mg), AOT (Sodium bis-2-ethylhexyl sulphosuccinate) were obtained from Sigma Chemicals Co. Ltd. (Poole, Dorset). *Micrococcus lysodeikticus* cells and ethylene glycol chitin substrates, reduced glutathione (GSH) and oxidixed glutathione (GSSG) were purchased from Aldrich Chemicals. Isooctane (2,2,4-trimethylpentane) was of AnalaR grade and purchased from BDH Chemicals (Merck Ltd., Poole, Dorset). Guanidine hydrochloride (GuHCl), urea and all the other alkanes used ranging from hexane to hexadecane were purchased from Aldrich chemicals. Riedel-de-Haen hydranal titrants (Coulomat A and Coulomat C) for Karl Fischer titration were purchased from Fisons Chemicals (Fisons scientific equipment, Loughborough). All water used for the experimental work was obtained from a reverse osmosis water unit (Elgastat Prima).

3.2 Experimental Methods

The majority of work in this thesis involved transferring lysozyme (native and non-native) from an aqueous phase to an organic phase containing the surfactant (forward transfer) and then recovering the protein from the organic phase back into a fresh aqueous phase (backward transfer) (Figure 3.1). The forward transfer of lysozyme was achieved by mixing equal volumes of an aqueous phase containing the protein with an organic phase containing the surfactant in stoppered vials on a rotary mixer. Once lysozyme had been transferred into the micelles in the organic phase, A_{280} measurements in the aqueous and organic phases revealed the quantity of transferred protein. Similarly for the backward transfer, the micellar phase now containing the protein was mixed with the same volume of an aqueous phase containing 1 M KCl. Again A_{280} measurements of both phases revealed the amount

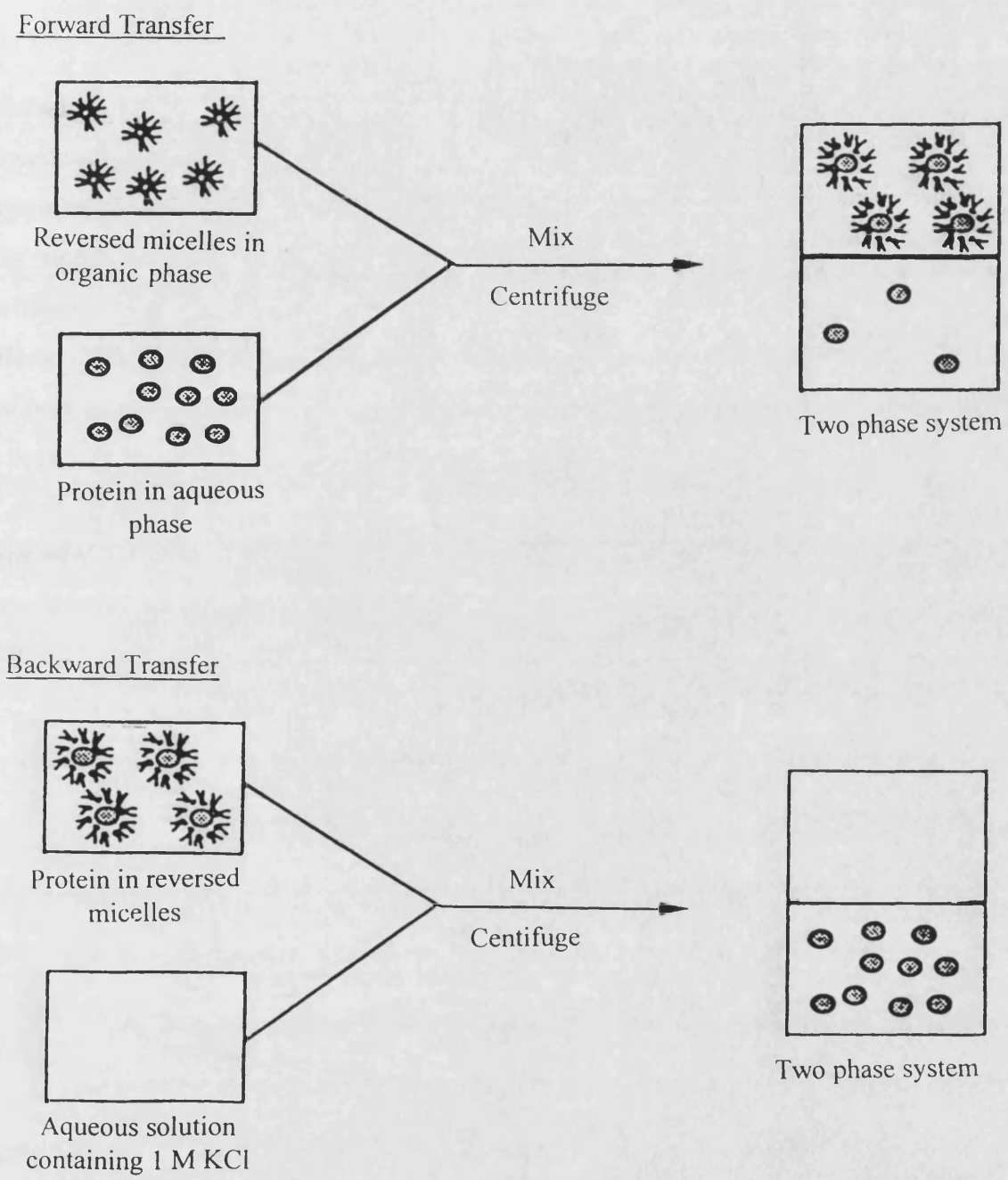

Figure 3.1 The forward and backward transfer of proteins

of protein recovered. More details of these transfer methods are given below.

3.2.1 Forward transfer of lysozyme

3.2.1.1 Transfer of native lysozyme

The forward extraction of lysozyme involved mixing equal volumes (5-10 ml) of an organic phase (50-400 mM AOT/isooctane) with an aqueous phase (1 mg/ml lysozyme in 25 mM phosphate buffer at a particular pH containing 0.1 M KCl) in tightly stoppered vials to allow protein transfer into the organic phase. In experiments using unbuffered aqueous phases, the pH was adjusted using 5 M NaOH or HCl. The amount of protein transferred was determined relative to the initial protein concentration present in the aqueous phase (1 mg/ml). The contact time used was 15 min unless otherwise stated. Whenever samples appeared cloudy they were centrifuged at 2000 rpm for 10 min to give two clear distinct phases. Mixing of the aqueous and organic phases for the forward and backward transfer processes was carried out using a rotary mixer (Stuart Scientific Rotary Drive STR4) at 56 rpm.

3.2.1.2 Transfer of denatured lysozyme

In the case for the non-reduced form of lysozyme, the protein was simply added to the denaturant solution (GuHCl, GuHCN or urea) at a particular concentration (1-10 M). For the reduced form of the protein, see section 3.2.6.1. The forward extraction of lysozyme in the denaturants GuHCl, GuHCN and urea was performed as above using 50 or 400 mM AOT/isooctane. When GuHCl and GuHCN were used no salt was present in the buffer (Tris-HCl, pH 8.7) due to the charge of the guanidinium ion. However, lysozyme denatured in urea contained 0.1 M KCl unless otherwise stated.

3.2.1.3 Transfer of lysozyme using mixed micellar systems

The forward extraction of lysozyme with the mixed surfactant systems involved mixing equal volumes (5-10 ml) of an organic phase (50-400 mM AOT/Tween 85 or AOT/Tween 20) with an aqueous phase (1 mg/ml lysozyme in various GuHCl (0-3 M) and KCl (0-3 M) concentrations in 25 mM phosphate buffer, pH 7.0) for 15 min. Lysozyme concentration in the aqueous and organic phases were determined spectophotometrically at 280 nm.

3.2.1.4 Transfer of lysozyme using mixed denaturant systems

The forward extraction of lysozyme with the mixed denaturant systems at different molar ratios (GuHCl:urea, 0.2:4.8, 0.3:4.7, 0.5:4.5, 1:4, 2:3, 1:1) involved mixing equal volumes (5-10 ml) of an organic phase (50-400 mM AOT/isooctane) with an aqueous phase (1 mg/ml lysozyme in various mixed denaturant systems in Tris-HCl, pH 8.7) for 15 min. Lysozyme concentration were determined in the aqueous and organic phases spectrophotometrically at 280 nm.

3.2.2 Backward transfer

3.2.2.1 The use of high pH

The backward extraction of lysozyme was achieved by mixing equal volumes (4-8 ml) of an aqueous phase containing 1 M KCl in Tris-HCl, pH 11.2 with the organic phase containing the protein from the forward transfer. The forward transfer experiments prior to the backward transfer were carried out at pH 7.0 containing 0.1 M KCl. The contact time used was 30 minutes unless otherwise stated.

3.2.2.2 The use of ethyl acetate

Equal volumes (4-8 ml) of the reversed micelle solution containing the protein from the forward transfer were mixed with an aqueous phase consisting of 1 M

KCl in 0.1 M Tris-HCl, pH 8.7 containing 0.1 ml ethyl acetate/ml. The samples were mixed for 30 min (unless otherwise stated) at room temperature and then centrifuged using a Koolspin model at 2000 rpm for 10 mins. The phases were then separated and analysed.

3.2.2.3 The use of isopropyl alcohol

This method was adapted from Carlson and Nagarajan (1992). Equal volumes (4-8 ml) of the reversed micelle solution containing the protein from the forward transfer were mixed with an aqueous phase consisting of 1 M KCl in 0.1 M Tris-HCl buffer pH 8.7, containing 5-50 % by volume isopropyl alcohol. The samples were mixed for 15 minutes at room temperature and then centrifuged at 2000 rpm for 10 min. The phases were separated (3.2.3) and analysed.

3.2.3 Phase separations for forward and backward transfer experiments

The phases were allowed to separate under gravity for 10-15 minutes in a water bath at 25°C. In the majority of the experiments the phase separations seemed to occur over a few seconds. In the experiments where two phase systems failed to give two clear phases the samples were centrifuged at 2000 rpm for 5 minutes.

3.2.4 Construction of phase diagrams

The reversed micelle system used within this project (AOT/isooctane) was monitored over a range of temperatures where an optically clear and homogeneous solution was formed as a function of added water, expressed as the mole ratio of water to surfactant (Wo value). The phase behaviour diagrams were constructed for the protein and non-protein reversed micellar system. A range of Wo values (10-60) were made up by adding 50-400 µl of distilled water to 5 ml of 50 mM AOT/isooctane and shaking manually. They were then placed in a thermostatted water bath and left to equilibrate at a chosen temperature. The temperature of the

water bath could be controlled to ±1°C. The temperature was changed over 2°C intervals between readings, typically at a rate of 1°C in approximately 5 minutes. Measurements were taken between 0°C and 70°C. The solutions were regularly given a vigorous shake and were observed visually to determine whether they were single- or multi-phased. A stable system was seen as being homogeneous and showing no signs of turbidity. The nature of the samples (one or multi-phased) were noted and the data plotted graphically to show the phase behaviour of the systems.

3.2.5 Refolding lysozyme inside reversed micelles

3.2.5.1 Reduction of denaturant concentration inside the reversed micelles by dilution

To remove the denaturants GuHCl and urea from the reversed micelles a number of contacting stages (1-10) were performed. These involved mixing 5-10 ml of the reversed micelle phase containing the denatured and reduced protein with the same volume of an aqueous phase solution containing 0.1 M KCl or 0.1 M NaCl in 0.1 M Tris-HCl, pH 8.7. The samples were mixed for 15 min and then centrifuged at 2000 rpm for 15 min giving two clear phases. This step was repeated as necessary with the resulting organic phase and a fresh aqueous solution in order to remove any additional denaturant. After each contacting stage, the protein content and water content were determined.

3.2.5.2 Glutathione addition for disulphide bond formation of lysozyme inside reversed micelles

Stock 40 mM solutions of reduced (GSH) and oxidised (GSSG) glutathione were made up in 0.1 M Tris-HCl, pH 8.7. These were mixed in a number of different ratios (between 1:1 and 10:1) of the reduced to the oxidised form and 50-100 µl of this was injected into 1 ml of 50 or 400 mM AOT/isooctane solution.

3.2.6 Refolding lysozyme in the aqueous environment

3.2.6.1 Reduction of lysozyme

Protein solutions (20 mg/ml) in 0.1 M Tris-HCl, pH 8.6 containing 6 M GuHCl (unless otherwise stated) and 0.15 M dithioerythritol were incubated for 2 hr at 20°C. The solutions were then acidified to pH 3 by the addition of 5 M HCl and dialysed extensively against 0.1 M acetic acid at 4°C for 12-14 hr. The protein was verified to have all 4 disulphide bonds broken prior to any refolding experiments (3.3.3).

3.2.6.2 Inactivation of lysozyme in GuHCl

Lysozyme (0.5 mg/ml) was incubated in 0.06 M potassium phosphate, pH 6.2 in the absence and presence of increasing concentrations of GuHCl at 20°C for 15 min. Enzyme activity was assayed in the same concentration of GuHCl and expressed relative to a control sample from which GuHCl was omitted.

3.2.6.3 Reactivation of lysozyme in an aqueous environment

Renaturation-reoxidation of the enzyme solution was initiated by adding a sample of enzyme to renaturation buffer (0.1 M Tris-HCl, pH 8.2, 1 mM EDTA, 3 mM reduced glutathione and 0.3 mM oxidised glutathione), mixing with a vortex mixer for 15 s and incubating at 40°C. Enzyme activity was assayed at several points and expressed relative to native lysozyme measured at the same concentration.

3.3 Analytical Methods

This section describes a number of analytical methods that were constantly used throughout the project. Protein concentrations in the aqueous and organic phases

were determined by absorbance spectrophotometry. Karl-Fischer titration was used to evaluate the water content inside reversed micelles which could then be related to the size of these droplets. The structural analysis of lysozyme was obtained using the Ellman assay which monitors the state of the disulphide bonds and *Micrococcus lysodeikticus* which was used to measure the activity of the protein. Circular dichroism (CD) and fluorescence were also used to study the structural state of the protein in and outside reversed micelles. More detail of these methods are given below.

3.3.1 Measurement of protein concentration

The concentration of protein in solution was determined using a Cecil 3000 series scanning spectrophotometer (Cecil instruments). Lysozyme concentrations were determined spectrophotometrically at 280 nm using an absorbance for 1 mg/ml of 2.63 for native lysozyme and 2.37 for denatured lysozyme (cell path length = 1 cm), Saxena and Wetlaufer (1970).

3.3.1.1 Measurement of protein concentration in the aqueous phase

Lysozyme concentration was measured in the aqueous and organic phase at 280 nm for both the forward and backward extractions. The molar extinction coefficient (ε_{aq}) for lysozyme at 280 nm in the aqueous phase is 36036 $cm^{-1}M^{-1}$, (Lye, 1993).

3.3.1.2 The Folin-Lowry assay

Occasionally the measurement of protein concentration in the aqueous phase was difficult due to the solutions becoming cloudy. The cloudiness was observed after the forward transfer of lysozyme from the aqueous to the organic phase. In these cases the Bio-Rad assay which is a modified version of the Folin-Lowry assay for

determining protein concentration was used as it allows the measurement of protein concentrations in the presence of surfactants.

3.3.1.3 Measurement of protein concentration in the organic phase

The protein solution (1mg/ml) was injected directly into the organic phase (50 mM AOT/isooctane) which ensured that all the protein was present in the organic phase. A 40 mg/ml lysozyme solution was prepared by dissolving 40 mg of lysozyme in 1 ml of 20 mM phosphate buffer, pH 7. 100 μl of this solution was directly injected into 4 ml of 50 mM AOT/isooctane to give a final concentration of 1 mg/ml. The lysozyme solution was diluted to prepare a range of standards and the absorbance at 280 nm was measured for each standard. The molar extinction coefficient (ε_{rm}) for lysozyme at 280 nm in the organic phase is 36894 $cm^{-1}M^{-1}$, Lye (1993).

3.3.1.4 Mass balance for the quantification of precipitate

Whenever lysozyme (1mg/ml) was transferred from the aqueous phase to the organic phase or vice-versa, the protein could be distributed between both of the phases or be present at the interface. The protein concentrations were determined for the organic and aqueous phases, the concentrations were combined and when the result did not equal 1 mg/ml then the rest of the protein was assumed to be present at the interface. Equation 3.1 can be used to determine in which phase lysozyme was present in all transfer experiments

$$[C_{Lys} V_{aq}] = [C_{rm} V_{aq} + C_i V_i + C_{aq} V_{aq}] \qquad (3.1)$$

where C_{Lys} is the total lysozyme concentration, V_{aq} is the volume of the aqueous phase, C_{rm} is the concentration of lysozyme in the reversed micelle, C_i is the concentration of the protein at the interface and V_i is the volume at the interface. All protein transfer results were evaluated using this technique.

3.3.2 Measurement of AOT concentration in aqueous and organic phases

Surfactant concentrations were determined by a two-phase titration. The measurements of aqueous phase AOT concentrations were performed by adding 1-2 drops of phenolphthalein solution to a 10 ml sample followed by a few drops of 1 M NaOH until the solution turned pink in colour. To this solution 5 ml of distilled water was added followed by 7.5 ml chloroform and 5 ml of the acid indicator solution (dimidium bromide-disulphide blue). At this stage the pink colour is transferred to the chloroform layer. The solution is now titrated with 0.004 M hyamine solution shaking vigorously after each titration step. This step is continued until the the pink colour from the chloroform phase becomes clear indicating the endpoint of the titration. If excess hyamine is added, the solution turns dark blue in colour. The volume of the titrant was noted.

The same method was employed for AOT determinations in the organic phase except that 200 µl was diluted to 10 ml with isooctane. The addition of phenolphtalein and the other reagents were performed as before.

3.3.3 Ellman assay for the state of disulphide bonds

The ellman assay measures the nitrothiobenzoate (NTB) released upon reaction of a thiol with DTNB. This method has been adapted form Creighton (1990). A protein solution (1 mg/ml) was prepared in 6 M GuHCl with 0.1 M phosphate buffer (pH 3) and 1 mM EDTA. A blank for the reference was also prepared containing exactly the same without the protein. To 1 ml of sample, 50 µl of 3 mM DTNB (in 0.1 M phosphate buffer, pH 3.0) was added and thoroughly mixed. The increase in absorbance was monitored of the protein solution at 412 nm. After

absorbance measurements stopped increasing the reaction was complete and the reference subtracted. From the increased absorbance in the protein sample caused by the DTNB, the molar concentration of thiols present from the molar absorbance of the TNB anion were calculated.

3.3.4 Lysozyme activity assay

Lysozyme activity was measured using the substrate *Micrococcus lysodeikticus.* Activity measurements were performed on the aqueous phase containing lysozyme after the back transfer using a suspension of *Micrococcus lysodeikticus.* In the assay lysozyme cleaves bacterial cell walls by hydrolysing glycosidic bonds. The turbidity of a bacterial cell suspension is thereby reduced to an extent proportional to the catalytic activity of lysozyme in the assay mixture.

Lysozyme activity was determined at 25°C by following the decrease in absorbance at 450 nm of a 0.25 mg/ml *Micrococcus lysodeikticus* suspension in 0.06 M potassium phosphate, pH 6.2. The assay volume was 1 ml and one unit of enzyme activity corresponds to an absorbance decrease of 0.0026 per minute, Goldberg *et al.* (1991).

3.3.5 Karl-Fischer titration for the analysis of water content

Karl-Fischer titration was employed to determine the water content present within reversed micelles in the organic phase. In this coulometric technique, iodine is electrolytically generated in the anolyte which contains iodide. When water is present in the titration vessel the iodide reacts with it.

$$H_2O + I_2 + SO_2 + CH_3OH + 3RN \rightarrow [RNH]\,SO_4CH_3 + 2[RNH]I \quad (3.2)$$

$$2I^- \rightarrow I_2 + 2e^- \quad (3.3)$$

Water reacts with iodine and sulphur dioxide in the presence of a base alcohol (Equation 3.2). Once all the water has reacted a small excess of iodine appears in the anolyte. The iodine is detected by a double platinum pin electrode and iodine production is stopped. From Faraday's law the quantity of iodine produced is proportional to the current generated. So the total amount of moisture can be determined by measuring the total consumption of electricity.

Samples (0.05-0.1 ml) were weighed in a syringe and then injected via a septum. The parameter known as Wo is used to determine the size of the reversed micelle.

$$\mathbf{Wo = [H_2O] / [Surf]} \qquad \mathbf{(3.4)}$$

The instrument measures the amount of water in the sample as a percentage (w/v) which can then be converted to Wo by the formula shown below :-

$$\mathbf{Wo = 7.69 \times \% \text{ water}} \qquad \mathbf{(3.5)}$$

The results obtained were an average of three determinations.

3.3.6 Protein Conformation

Two methods have been chosen to study the structure of lysozyme before and after extractions into and out of the reversed micelles. Fluorescence and circular dichroism (CD) have been chosen to look at the conformation of lysozyme.

3.3.6.1 Fluorescence Spectroscopy

Fluorescence measurements were made at 20°C in a Perkin-Elmer LS-5B fluorimeter. The excitation wavelength was 290 nm and the emission spectra between 300 and 400 nm were recorded after the addition of lysozyme to the various ratios of denaturants. Lysozyme was incubated at 10 μg/ml for 30 min before spectra were recorded.

3.3.6.2 Circular Dichroism

CD measurements were made at 25°C in a Jobin Yvon CD 6 instrument. Lysozyme was denatured in GuHCl, urea and a mixture of these denaturants in various ratios at particular concentrations (1-10 M) in Tris-HCl, pH 8.7. Lysozyme concentration in the aqueous and organic phases were normally 1 mg/ml unless otherwise stated. A 3 ml sample volume was required for the analysis of tertiary structure of the protein in the near-uv region. The CD spectra were recorded between 250 and 320 nm and the tertiary structure of the protein determined. Each spectrum was an average of three readings and in all cases the relevant background was subtracted . The scan rate used was 2 nm/sec.

CD measurements were not performed in the far-uv region where there is absorbance of isooctane as well as the denaturants GuHCl and urea. Therefore CD use was restricted to the wavelength region above 220 nm.

CHAPTER 4

THE FORWARD TRANSFER OF NATIVE AND NON-NATIVE LYSOZYME INTO AOT/ISOOCTANE REVERSED MICELLES

This chapter describes the forward transfer of native, non-reduced and reduced lysozyme into AOT/isooctane reversed micelles. In each case parameters such as pH, ionic strength and surfactant concentration have been varied in order to optimize the forward transfer of lysozyme. The transfer of lysozyme has also been investigated using a mixed micelle system and a mixed denaturant system in order to transfer lysozyme at higher denaturant concentrations. These conditions need to be established and optimized in the early stages of the overall process. These will identify the processes that control protein solubilisation in reversed micelle systems. This understanding that may be gained by studying single proteins in micellar solutions may permit the process to be employed as a refolding technique and the appropriate conditions required can then be determined.

4.1 Phase behaviour studies

Phase behaviour studies provide an insight into the conditions that show the stability of the interactions that occur within reversed micellar systems. Using this approach it is possible to gain a full understanding of the factors which influence reversed micelle stability and appreciate the conditions which favour the formation of these nanometer-sized droplets. Two systems were studied, the first was the AOT/water/isooctane system and the second was the addition of lysozyme to the water pool of this system.

4.1.1 Phase behaviour for a 50 mM AOT/water/isooctane system

The preparation of the samples of differing Wo and the experimental procedure are described in Chapter 3. Wo is a measure of the size of reversed micelles formed and has been described in Chapter 2. Figure 4.1 shows the phase behaviour of a 50 mM AOT/water/isoctane system.

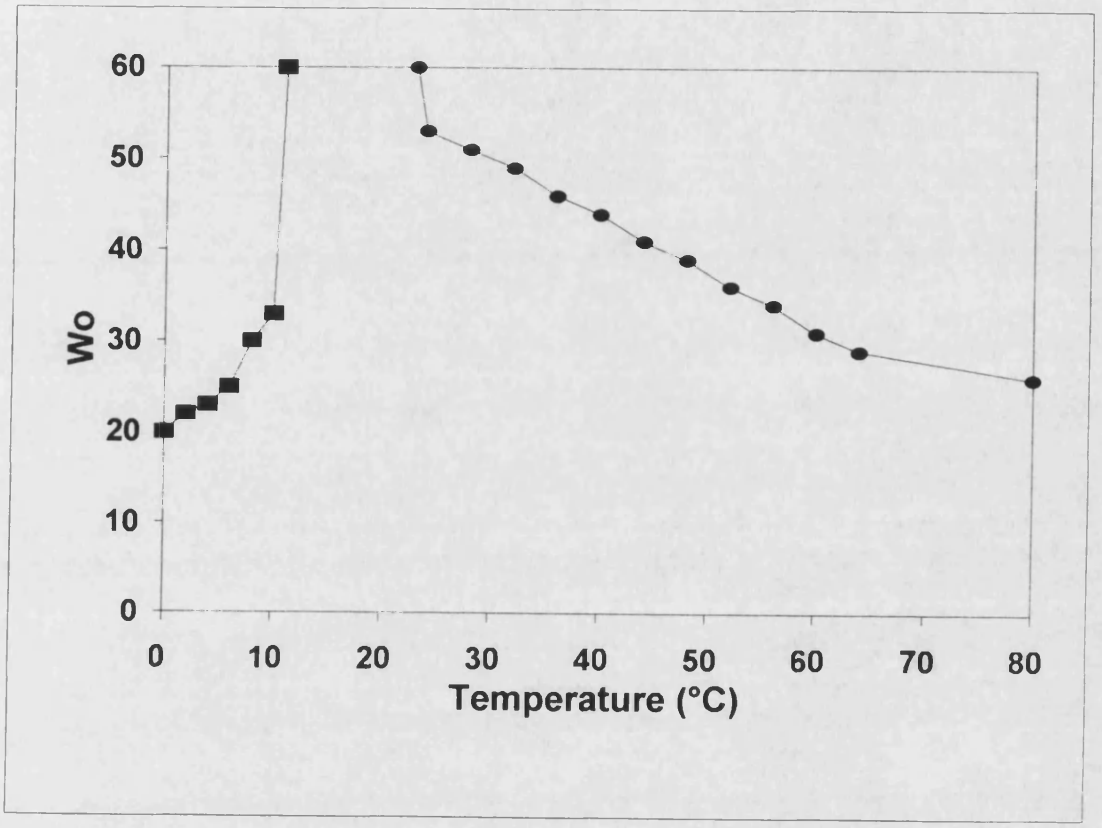

Figure 4.1. Wo vs temperature plot for the 50 mM AOT/water/isooctane system.

A range of Wo values were made up by mixing various amounts (20-200 µl) of distilled water in 50 mM AOT/isooctane. The samples were shaken manually to give a clear one phase solution and then incubated in a thermostated water bath for 15 min to equilibrate. Measurements were taken from 0-80°C and the phase diagram was constructed by visually noting whether the samples were one or two phases. The stable region was formed between the lower phase boundary (■) and the upper phase boundary (●).

There are two distinct boundaries of the graph: the upper and lower phase boundary. Above these boundaries eg at a temperature of 5°C and a Wo value of 36 and 50°C and a Wo of 48 a two phase region exists showing the instability of this particular system. Between and below the two boundaries, a single phase stable microemulsion is formed. As the temperature is increased from the two phase region, through the lower temperature phase boundary, a single phase system is formed consisting of discrete, nanometer-sized water droplets that are stabilized by a monolayer of surfactant. The positions of these boundaries are independent of AOT concentration (at a fixed Wo) since the AOT does not significantly partition from the water-oil interface to the oil. As the temperature is increased and approaches the upper phase boundary, the attractive interactions between droplets increase and the phase boundary is reached when the inter-droplet interactions become irreversible. At this boundary, the initial one-phase system separates into two phases: a dilute droplet phase consisting mostly of oil and water and a surfactant rich phase (Kotlarchyk *et al.*, 1984). The strength of the attractive interactions present in the one phase region, increases with droplet size, or Wo, so the temperature range of the single-phase droplet region, decreases at higher Wo (Brunetti *et al.*, 1983). The upper-temperature phase transition is due to changes in solvation of the surfactant tail-groups by the oil (isooctane) present in the system, an increase in temperature causing de-solvation of the surfactant tail-group by the oil (Huang, 1985).

The shape of phase stability plots produced depend on the property of the surfactant used within a system and it is expected that geometrical structural properties will be important. Mall (1992) has studied the phase behaviour for a number of di- and tri- chain surfactants. The basic structure of these plots are similar to that shown in Figure 4.1. There are many important parameters which can change the structure of the phase stability plots and one of these is the oil solvent variation. Fletcher *et al.* (1987) have determined the single phase microemulsion regions for different n-alkane solvents in 0.1 M AOT and found that as the alkane chain length increases, the region of microemulsion stability is shifted to lower temperatures.

4.1.2 Phase behaviour for a 50 mM AOT/water + protein/isooctane system

The phase diagram for a 50 mM AOT/water/isooctane system was constructed and the stable regions for microemulsion formation determined. The next step is to see how this stability plot might change by incorporation of lysozyme into the system. Figure 4.2 shows how the presence of lysozyme (1 mg/ml) in the water pool of the microemulsion changes the phase diagram. The lower phase boundary which was present at 10°C (Figure 4.1) is now in the presence of lysozyme at 30°C, corresponding to a 20°C shift. The upper phase boundary also shifted to a higher temperature by about 30°C, thus broadening the whole single-phase area with respect to temperature and Wo values. The result of this phase stability plot indicates that by adding lysozyme to these reversed micellar systems changes are observed in the shape and extent of the one-phase microemulsion region. Possible protein-induced effects on microemulsion droplets include: direct surfactant-protein interactions (Kotlarchyk *et al.*, 1984), adsorption of the protein at the water/oil interface (Greener *et al.*, 1987) or the binding of the surfactant counterion which may affect the head group packing (Luisi and Agnew, 1985).

The results presented in this section allow one to identify the range of temperatures and Wo values over which a stable reversed micellar system can be formed. For both the non-protein and protein containing micellar system there exists a lower and upper-temperature phase boundary. Between the two boundaries a stable reversed micellar system exists since a clear thermodynamically stable solution is formed. Above and outside the two phase boundaries, the system can be seen to coalesce into two distinct phases at a given temperature. The addition of lysozyme to the system increased the Wo and temperature range over which a stable reversed micellar system was formed. These results enable the temperature range and Wo used throughout this thesis to be confined to values where the reversed micellar system is stable.

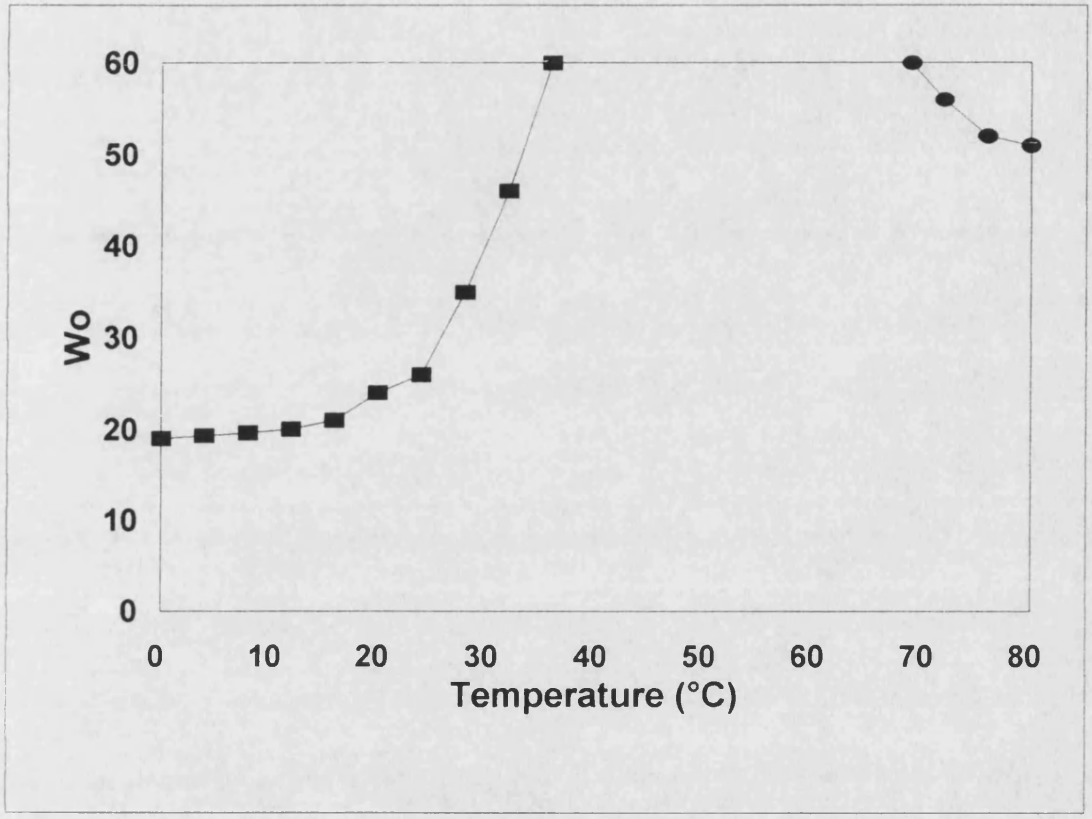

Figure 4.2. Wo vs temperature plot for the 50 mM AOT/water + protein (1 mg/ml)/isooctane system

A range of Wo values were made by mixing various amounts (20-200 μl) of distilled water containing 1 mg/ml lysoyzme in 50 mM AOT/isooctane. The procedure was repeated as for Figure 4.1. The stable region was formed between the lower phase boundary (■) and the upper phase boundary (●).

4.2 Forward transfer of native lysozyme

In this section the effects of pH, ionic strength, contact time, salts and surfactant concentration on the forward transfer of native lysozyme from an aqueous solution to an AOT/isooctane reversed micellar system are described. This should give an understanding of the factors which control the solubilisation behaviour of lysozyme by a systematic variation of system parameters. These factors can then be used to see what influence they have on the transfer of denatured lysozyme into reversed micelles.

4.2.1 The effect of pH on the forward transfer of lysozyme

The effect of pH on the forward transfer of lysozyme was investigated. The pH of the protein was maintained by using a potassium phosphate buffer. A low ionic strength was maintained by using a salt concentration of 0.1 M KCl throughout these experiments, unless otherwise stated. This low ionic strength is required to make the system stable. During the forward transfer the protein was sometimes observed as a precipitate at the aqueous/organic phase interface. By measuring the residual protein concentration in the aqueous phase and the concentration of protein in the organic phase, the mass of protein precipitated at the interface was quantified. Figure 4.3 shows that between pH 2-4 there was a large formation of precipitated protein at the interface. At these low pH values, little protein was partitioned into the organic phase. The protein at the interface was found to be in the form of a protein-surfactant precipitate. The maximum amount of protein transferred (90%) occurs over the pH range 7-11. Above pH 11 a sharp decline in the transfer of protein was observed, this coincides with the isoelectric point of lysozyme (pI=10.9).

In the low pH range (2-4), lysozyme may have partially unfolded and exposed some of its hydrophobic patches. These could then interact with the surfactant and form the precipitate at the interface. This will be discussed fully in Section 4.2.1.1 of the thesis. It is possible that lysozyme experienced some denaturation at the

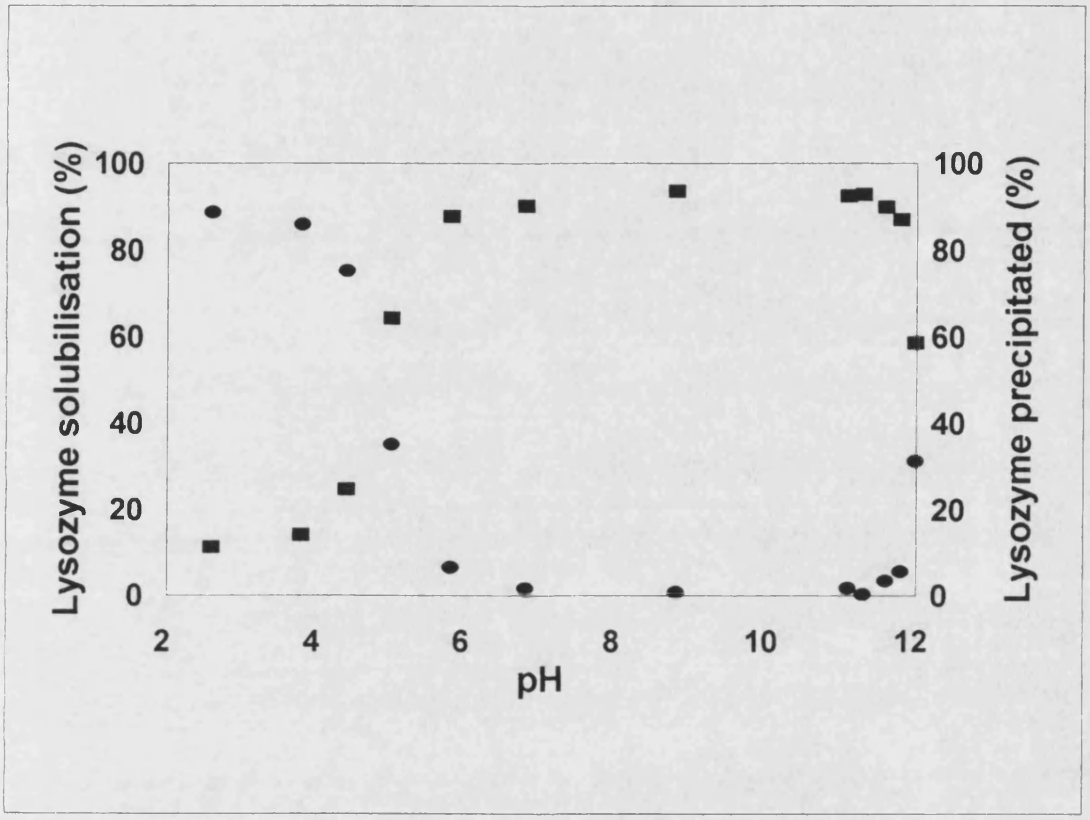

Figure 4.3. The effect of pH on the forward transfer of lysozyme

The forward extraction of lysozyme involved mixing equal volumes (5 ml) of 50 mM AOT/isooctane with an aqueous phase (1 mg/ml protein containing 0.1 M potassium chloride in 25 mM phosphate buffer at a particular pH (2-12) for 15 min. The resulting two cloudy phases were centrifuged at 2000 rpm for 15 min to give two clear phases. The protein concentration of both phases was measured and the amount of protein solubilised (■) and precipitated (●) was determined.

lower pH values which could have resulted in the transition to a random coil structure, which could be size excluded from the micelle giving the poor transfer observed. Since the protein is far from its isoelectric point (10.9) at these acidic values, the protein has a lot of positive charges which could lead to intramolecular repulsion and contribute to unfolding (Fink *et al.* 1990). The extent of precipitate formation was determined by a simple mass balance. Göklen and Hatton (1987) and Nishiki *et al.* (1993) studied the effect of pH on the forward transfer of lysozyme into AOT/isooctane micelles and obtained similar results but the amount of precipitate formed at the interface was not quantified.

When the pH of the solution is less than the pI of lysozyme then the protein has a net positive charge. As the surfactant headgroups of AOT possess negative charges, the protein is extracted into the micelle due to these favourable electrostatic attractions. However, when the pH of the solution exceeds the pI, the protein is negatively charged and is repelled out of the micelle which results in a decrease in transfer. It is quite clear from these results, that for a protein to be solubilised in reversed micelles of an anionic surfactant, the extraction pH must be below the pI of the protein. Similarly, for proteins to be extracted into cationic micelles, the pH of the solution should be greater than the pI of the protein (Matzke *et al.*, 1992). These results indicate that electrostatic interactions are necessary for protein solubilisation.

In conclusion, the aqueous phase pH determines the ionisation state of the surface charged groups on the lysozyme molecule. Attractive electrostatic interactions between the protein and AOT head groups occur if the overall charge of the protein is opposite to that of the surfactant head groups. Lysozyme was solubilised (90%) effectively in these conditions since the net charge on the protein was opposite that of the reversed micellar interface. However, when the overall charge of the protein is the same as the surfactant head groups very little protein is transferred and these conditions can be used for the back extraction step (Section 6.2.1). Lysozyme was also observed to denature at low pH values (<3) which was visually observed as a precipitate at the interface. This can be explained by

lysozyme unfolding at these low pH values and allowing exposed hydrophobic patches to interact with the surfactant forming a precipitate.

4.2.1.1 The protein/surfactant complex

The lysozyme that precipitated at the aqueous/organic interface at low pH values (Section 4.2.1) was believed to occur due to unfolding of the protein molecule at these acidic values. The nature of this precipitate is assumed to be a complex of denatured protein and surfactant. Göklen and Hatton (1987) have also observed this when proteins were transferred into micelles under conditions outside their stable pH range. Using the two phase titration method (Chapter 3), this complex was analysed to determine how much surfactant was present. Between 2-5% of the AOT was found in the precipitate together with 80-90% of the protein which was determined using a mass balance between the organic and aqueous phases. AOT concentrations were also determined in the aqueous phase where no AOT was detected and the organic phase which contained 95% of the surfactant. Again the mass balance of the AOT between the two phases proved that 5% of the surfactant was found in the precipitate.

The protein/surfactant complex formed at the interface could not be dissociated and hence the protein could not be recovered suggesting that the protein irreversibly binds to the surfactant. Lye (1995) has obtained similar results on this protein/surfactant complex and was also unable to dissociate it.

4.2.1.2 Conformational studies of lysozyme in reversed micelles using near-uv CD

The structure of lysozyme in and out of AOT/isooctane reversed micelles has been investigated by near uv-CD measurements. Unfortunately CD data could not be recorded in the far-uv range for secondary structure especially for lysozyme inside the reversed micelle since in this region salts tend to have an absorbance spectrum which tend to dominate making it difficult to observe any secondary structure.

Also the small volume of organic phase required tended to evaporate over the time the spectra was recorded.

Figure 4.4 shows the near-uv CD spectra for native lysozyme both in and outside 50 mM AOT/isooctane reversed micelles. The native protein (1 mg/ml) in an aqueous solution shows the characteristic peak between 280-320 nm due to the tryptophan residues. The peak in this region is characteristic of proteins containing tryptophan and tyrosine residues and is commonly used to monitor 3D structure. However, after the protein is transferred into the micelles the peak is lost showing complete loss of tertiary structure. This is an interesting result in that the aqueous microenvironment provided by the reversed micelles has a denaturing effect on the protein.

Grandi *et al.* (1981) found that lysozyme solubilised in AOT/isooctane reversed micelles were active, especially at low water contents (0.8% water v/v). Their spectroscopic studies indicated that the conformation of lysozyme was significantly different in reversed micelles compared to its conformation in water. The CD studies showed that the helical content of lysozyme changed from 30% in water to 48% in the reversed micelles. There appears to be contradictory evidence regarding the conformation of lysozyme in reversed micelles as the CD data confirms some protein unfolding occurs in the microenvironment but Grandi *et al.* (1981) found that the protein retains activity. This can be explained by the fact that the native configuration of the protein is maintained when it is bound to its substrate. Inside the micelle in the absence of the substrate the protein unfolds as shown by the CD data presented. The results in Figure 4.4 show that the protein has a molar ellipticity of zero when transferred into the micelles. The active site of the protein must orient in a different way and since no substrate is present for the protein, the AOT head groups must have a denaturing effect. Steinmann *et al.* (1986) performed CD, fluorescence and NMR studies and hyothesised that in the absence of the substrate, the protein in AOT reversed micelles must undergo an

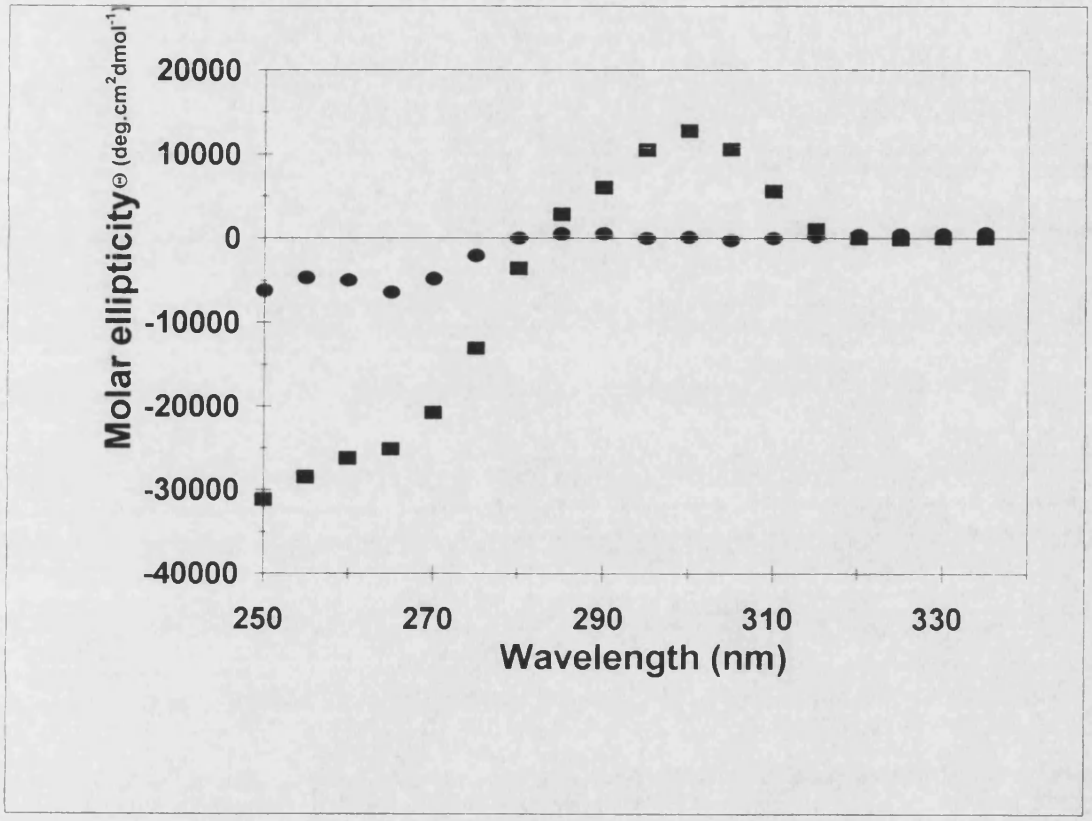

Figure 4.4. Circular dichroism spectra of lysozyme in and outside 50 mM AOT/isooctane micelles

Circular dichroism spectra in the near-uv were recorded for lysozyme in the aqueous environment (■) and inside 50 mM AOT/isooctane micelles (●). The samples were left for 30 min before scanning between 250-320 nm. The spectra were recorded at a scanning rate of 2 nm/min and are an average of 3 determinations after subtraction of the buffer blanks.

irreversible denaturation and this denaturation could be slowed down by the presence of a substrate.

The CD studies give a valuable insight into the structure of lysozyme inside and outside AOT/isooctane reversed micellar systems. In the aqueous solution the native three-dimensional structure of the protein was observed to have a prominent peak occurring at around 300 nm, which is characteristic of the tryptophan and tyrosine residues. However, on transferring the native protein from the aqueous solution to the organic phase the tertiary structure of the protein was lost as the peak at 300 nm gave a molar ellipticity value of zero. This means that the protein denatures inside the micelle possibly due to binding of the AOT head groups on the surface of the protein. The next chapter presents results on the transfer of denataured lysozyme into AOT/isooctane reversed micelles and how these may be different to those presented in this section.

4.2.2 The effect of contact time on the forward transfer of native lysozyme into 50 mM AOT/isooctane micelles

An important parameter in transferring proteins into reversed micelles is the contact time for distribution of the protein between the two phases. Figure 4.5 shows the effect of contact time using a rotary mixer (56 rpm) on the equilibrium distribution of lysozyme between an organic phase and an aqueous phase. The duration of mixing is shown to affect the amount of protein transferred into the organic phase. After 0.5 min about 40% of the protein was transferred into the reversed micelles leaving 60% in the aqueous phase as determined by A_{280} measurement. A minimum contact time of 1 min is required to achieve the highest protein transfer (> 90%). The amount of protein left in the aqueous phase was also measured and from these results the mass balance was calculated. The mass

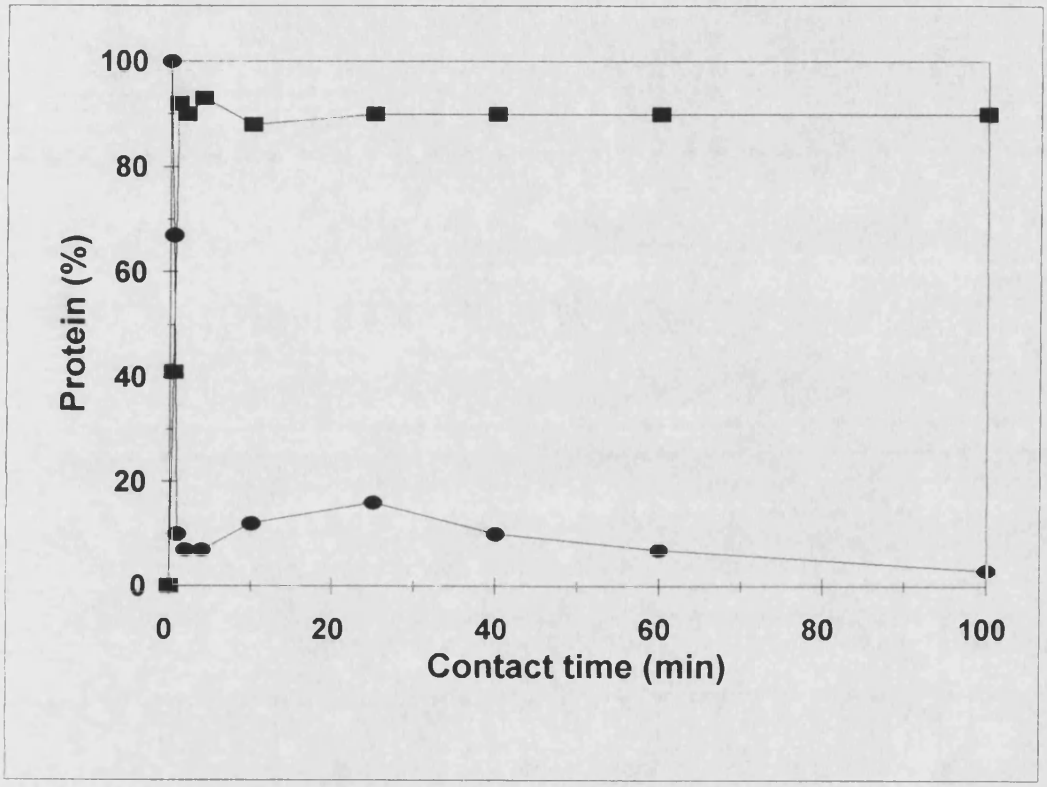

Figure 4.5. The effect of contact time on the forward transfer of lysozyme

The forward extraction of lysozyme involved mixing equal volumes (5 ml) of 50 mM AOT/isooctane with an aqueous phase (1 mg/ml lysozyme containing 0.1 M KCl in 25 mM phosphate buffer, pH 7.0) for various (0-100 min) times. The phases were separated by centrifugation at 2000 rpm for 10 min and the protein concentration determined in the organic phase (■) and the aqueous phase (●).

balance between the organic and aqueous phase gives a standard error of less than 3%. It should also be noted that precipitation at the interface did not occur in these transfer experiments, as the conditions used for these experiments (pH 7.0 aqueous solution containing 0.1 M KCl), were in the optimum range for transfer to occur with no formation of precipitate (Section 4.2.1). Marcozzi *et al.* (1991) found that the intensity and duration of mixing considerably affects the kinetics of α-chymotrypsin transfer. They showed that the time required for reaching the same equilibrium percentage transfer was around 6 h when mixing at 120 rpm and only 15 min at 350 rpm. The shorter time of 15 min was used for their work since it minimises the time in which the α-chymotrypsin could denature. All experiments involving forward extraction in this thesis used a contact time of 15 min unless otherwise stated.

The phase transfer method that was employed to transfer lysozyme is comparable to that of Kinugasa and Watanabe (1992) and Kinugasa *et al.* (1991). They found the phase transfer method to be successful for forward extractions of lysozyme. Generally, the forward extraction of proteins into micelles is a fast step and the rate of transfer is mainly controlled by the diffusion in the aqueous film and solubilisation at the interface (Nishiki *et al.*, 1993). Different mechanisms exist for the forward and backward transfer step and the forward transfer process is 10-1000 times faster. Other forward transfer mechanisms suggest that the process is determined by diffusion in the aqueous boundary layer (Kinugasa *et al.*, 1991) or interfacial solubilisation (Dungan *et al.*, 1991). The forward transfer mechanism has been proposed to be a cooperative process where the surfactant interface encapsulates the protein to form a reversed micelle, Dungan *et al.* (1991). The electrostatic interactions between the surfactant interface and the protein molecule show that the charges on the protein surface cause a deformation of the interface around the protein molecule. The extent of this deformation depends on both the distance between the protein and interface and also upon the aqueous phase pH and ionic strength. The rate of transfer will be protein and micelle specific and no generalisations may be made. It should be noticed that the purpose of this study

was to define a suitable contact time for the transfer of lysozyme from an aqueous phase to a reversed micellar phase.

In summary, the rate of transfer of lysozyme into a 50 mM AOT/isooctane system is a rapid process with more than 90% of the protein being displaced into the organic phase in 1 min. A mixing time of between 10-100 min showed no further increases in protein transfer. There was also no sign of interfacial precipitate, indicating that there were no protein/surfactant complexes being formed and no denaturation of the protein occurred.

4.2.3 The effect of ionic strength on the forward transfer of native lysozyme

Figure 4.6 shows the partitioning of lysozyme into 50 mM AOT/isooctane micelles with NaCl and KCl. The presence of the salts have a significant detrimental effect on protein transfer. Each of the salts show a similar trend for lysozyme transfer (Figure 4.6), but the decline in the amount of protein transfer occurs at much lower KCl concentrations. At very low salt concentrations (< 0.5 M) protein transfer (> 70%) into the reversed micelles is achieved with both salts. The optimum KCl concentration for lysozyme transfer occurred at 0.6 M with 72% transferred into the reversed micellar phase. There was then a sharp drop in protein transfer and at 1 M only 10% was found in the micellar phase. Göklen and Hatton (1987) have shown a similar effect of KCl concentration on the solubilisation of lysozyme, cytochrome c and ribonuclease in AOT/isooctane micelles. For NaCl the optimum concentration was found to occur at 1 M with 90% of the protein being transferred into the reversed micelles. A decrease in transfer then occurs giving a value of 30% at a concentration of 1.5 M.

The reason for this decrease in protein transfer is due to the electrostatic shielding of surfactant head-groups in the reversed micelle by the cations from the salt. These ions reduce the repulsion between individual headgroups of the AOT molecules causing the micelle to shrink in size and is therefore unable to

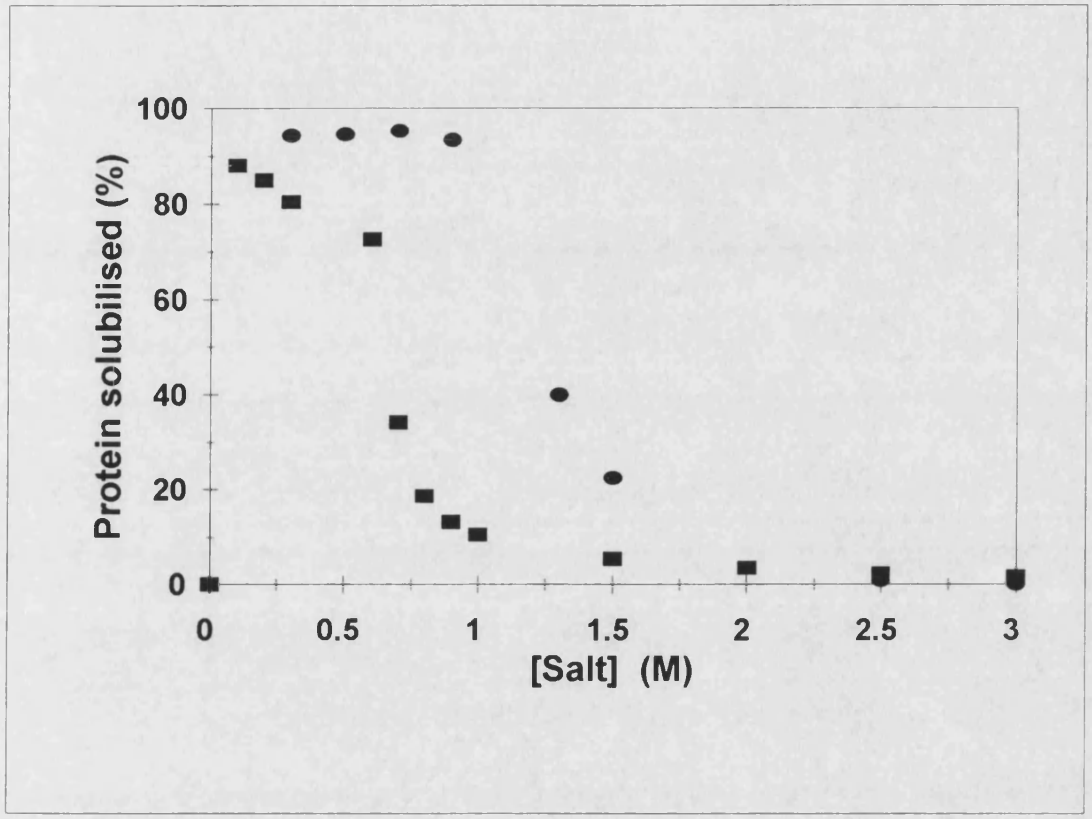

Figure 4.6. The effect of various salts on the forward transfer of lysozyme

Forward extraction experiments were performed by mixing equal volumes (5 ml) of 50 mM AOT/isooctane with an aqueous phase (1 mg/ml lysozyme in 25 mM phosphate buffer, pH 7) containing different salts (■ = KCl, ● = NaCl) at various (0-1.5 M) concentrations. The mixing time was 15 minutes at a speed of 56 rpm.

accommodate the protein. The main reason for the higher solubility of lysozyme at lower salt concentrations is that the water content of the reversed micelles increase at low ionic strengths and therefore they can accomodate more protein molecules. Marcozzi *et al.* (1987) have studied the factors affecting the forward and the backward transfer of α-chymotrypsin in AOT/isooctane micelles. They used four salts; KCl, NaCl, LiCl and $CaCl_2$ and found that the protein transferred at the lowest ionic strengths with KCl followed by $CaCl_2$, NaCl and LiCl. The atomic radii of these ions decrease in the order $K^+ > Ca^{2+} > Na^+ > Li^+$ showing that the size of these ions may also play an important role in these systems.

Different ions have differing propensities for adsorption onto charged surfaces. Smaller ions have a larger hydration shell and a low polarizability whereas larger ions of the same charge have a smaller hydration shell and greater polarizability. The larger ions have a greater tendancy to adsorb from solution onto an oppositely charged surface, forming the Stern layer, in which the electrostatic potential drops quickly in moving away from the surface of the charge (Figure 4.7). Ions of equal charge have been ordered in what is known as the Hofmeister series according to this observation. The diffuse double layer beyond this layer of adsorbed ions can be described by the Gouy Chapman model with the potential a function of the distance from the surface and the ionic strength of the solution. This situation can be seen in Figure 4.7. Different salts having the same ionic strength should be equally effective in screening the electrostatic potential in the diffuse double layer. However, different salts at equal bulk concentration or ionic strength will not be equally effective in screening the electrostatic potential in the Stern layer due to their different surface adsorption characteristics. Thus, it is apparent that the free energy of a system of two double layers, maintained at a constant separation from each other by a medium of a given ionic strength, will differ substantially depending on the salt used to obtain the desired ionic strength.

The Gouy Chapman model described above of the electrical double layer can be related to the inside of reversed micelles. This effect of salt type governs the solubilisation behaviour of proteins into reversed micelles. Results show that

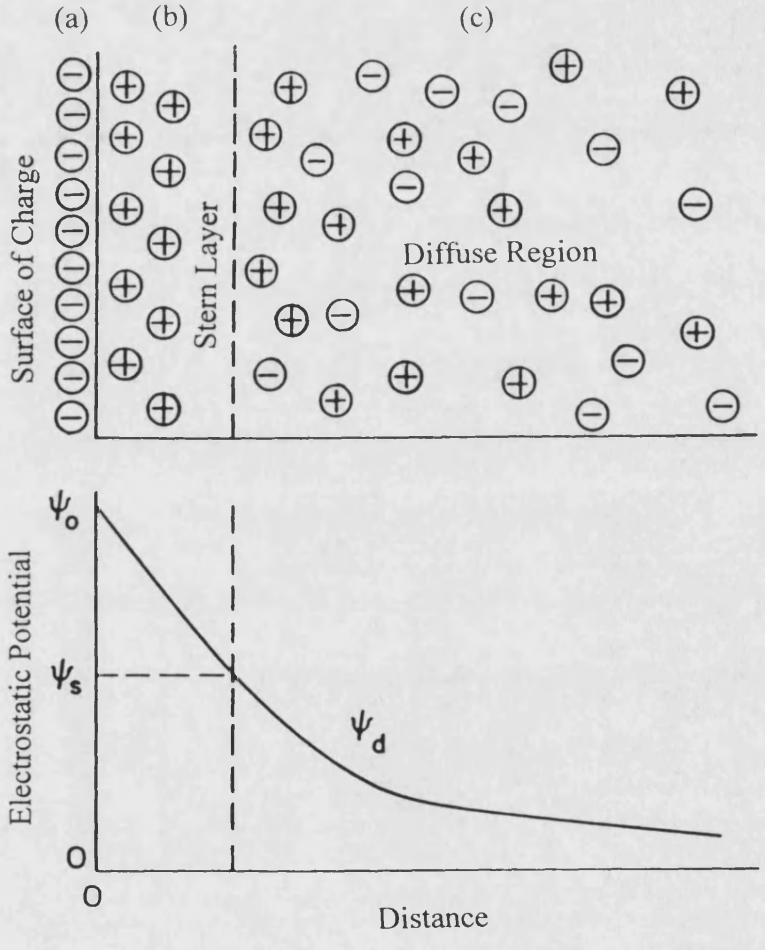

Figure 4.7 Electric double layer with a) Surface of charge, b) Stern layer of adsorbed ions and c) Diffuse double layer

increasing the ionic strength decreases protein transfer as at higher ionic strengths the electrical double layer would be compressed resulting in decreased head group repulsions. This means that ions which are more effective at compressing the double layer, due primarily to stronger adsorption at the surface of the charge, should yield smaller micelles and hence lower Wo values as will be shown in Section 4.3.1.1. For the two salts studied the diameter of K^+ (0.235 nm) > Na^+ (0.195 nm) and results show that potassium ions bind to a greater extent to the micellar wall than sodium ions so that a greater transfer is achieved with the sodium ion at higher concentrations.

Another important feature that should be noted is that different proteins have different responses to a given salt. A possible explanation for the different responses is that increases in ionic strength can affect the hydrophobic self-energy of a protein, Göklen (1987). The extent of this effect is dependent on the fraction of protein surface which is hydrophobic, which is not necessarily related to the size or the pI of the protein. Another possible explanation for this behaviour might be the difference in the way in which charge is distributed over the protein surface. Lysozyme transfer with KCl occurs over a much broader range (0-1 M) in comparison with α-chymotrypsin transfer with KCl (0.1-0.3 M), Marcozzi *et al.* (1987).

It has been shown that the forward transfer of lysozyme into AOT/isooctane reversed micelles is limited by the type and concentration of the salt. Ions having a large radii tend to adsorb more strongly to the negatively charged micellar interface than with ions with smaller radii. Also, the fact that smaller ions have a larger water of hydration associated with them means that the propensity to adsorb to the micellar interface is reduced. Results have shown that as the salt concentration is increased, the amount of protein transferred into the micelles was found to decrease for both salts studied. These positively charged ions bind to the micellar interface which in turn reduces the repulsive interactions between the surfactant head groups thus causing smaller micelles to be formed as will be shown in Section 4.3.1.1.

4.2.4 The effect of surfactant concentration on the forward transfer of native lysozyme

Another parameter in determining protein solubilisation in reversed micelles is the effect of surfactant concentration. Figure 4.8 shows how different AOT concentrations affect the solubilisation behaviour of lysozyme into micelles. At low surfactant concentrations (25 mM) the solubilisation region of the protein is much sharper than that obtained with 50 and 400 mM concentrations. The graph shows that at pH 4, 18% of the protein is transferred into the micelles at surfactant concentrations of 25 and 50 mM compared to 50% at 400 mM. In the lower pH range (2-6), the higher surfactant concentration of 400 mM appears to solubilise lysozyme more effectively than the lower surfactant concentrations. Between pH 7-10 there is no significant difference in the transfer of lysozyme with the surfactant concentrations used yielding 90% protein in the reversed micellar phase. However, at pH 11 the higher surfactant concentrations of 50 and 400 mM still yield 90% of the protein in the organic phase whereas for 25 mM the amount transferred drops to about 50%.

For AOT systems there is a minimum surfactant concentration for the solubilisation of lysozyme which corresponds approximately to the point where the surfactant can achieve monolayer coverage of the protein. Beyond this range, many surfactant aggregate structures are possible including micellar rods and various types of liquid crystals. In between these two ranges increasing the surfactant concentration results in larger spherical aggregates. It is clear that as the surfactant concentration increases, the range of pH values which lead to complete solubilisation of the protein is expanded. These results suggest that the surfactant concentration controls the size of the micelles and this is responsible for the solubilisation behaviour observed. Therefore, increasing the surfactant concentration leads to changes in micelle size and can be used to control solubilisation. This is an appealing prospect since larger micelles can be used to

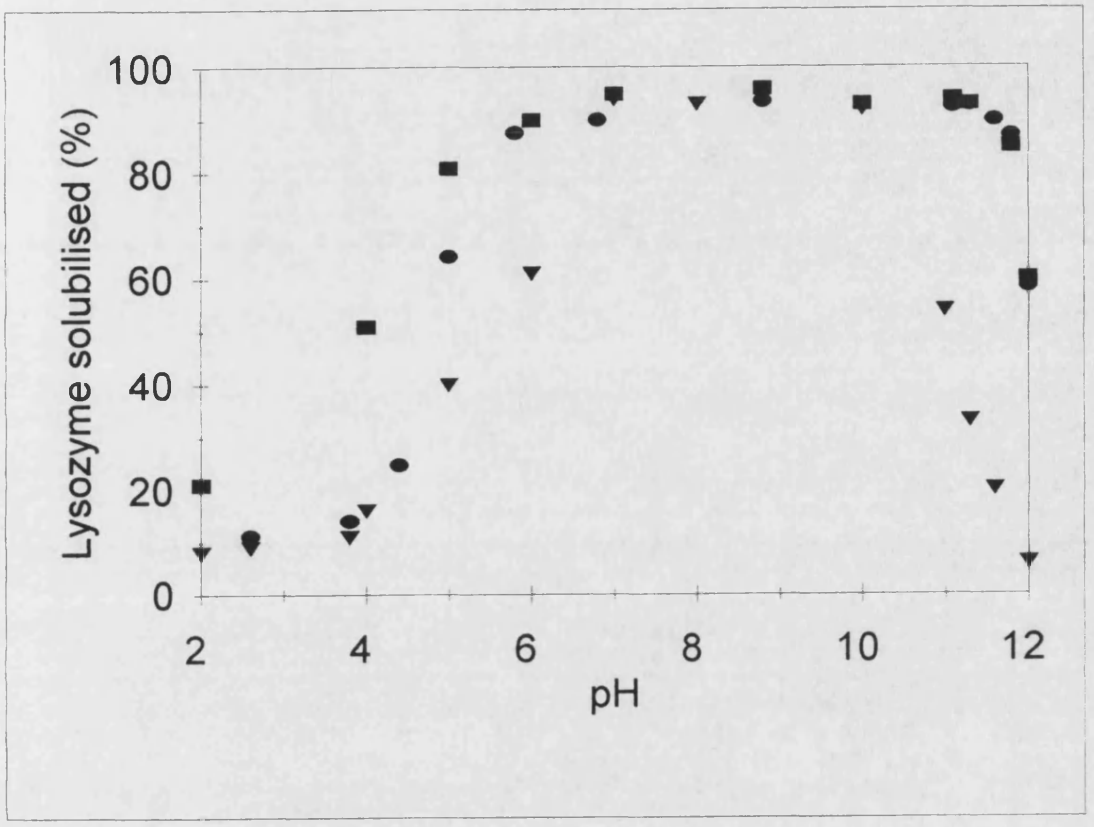

Figure 4.8. The effect of surfactant concentration on the forward transfer of lysozyme

The forward extraction of lysozyme involved mixing equal volumes (5 ml) of 25 mM (▼), 50 mM (●) and 400 mM (■) AOT/isooctane with an aqueous phase (1 mg/ml protein containing 0.1 M potassium chloride in 25 mM phosphate buffer at a particular pH (2-12) for 15 min). The resulting two cloudy phases were centrifuged at 2000 rpm for 15 min to give two clear phases. The protein concentration was determined in the organic phase.

solubilise larger proteins. Göklen (1986) has studied the solubilisation behaviour of a number of proteins into AOT/isooctane reversed micelles. He found that moderate increases in AOT concentration allows complete solubilisation of moderately sized proteins together with the broadening of the pH range over which complete solubilisation occurred. He postulated that increases in the equilibrium size of the micelles which occurred at the same time as increases in AOT concentration was responsible for the solubilisation of larger proteins.

In summary, it appears that increases in AOT concentration allow a broadening of the pH range over which solubilisation of lysozyme can be achieved. The increase in equilibrium micelle size with increased AOT concentrations is probably responsible for these observations. Concentrations above 400 mM were not used to investigate the transfer of lysozyme since the structure of surfactant aggregates at these concentrations is not well characterised and it is possible that lamellar structures will be formed.

4.2.5 Summary for the forward transfer of native lysozyme

The results presented above regarding pH, contact time, ionic strength and surfactant concentration appear to be the critical parameters required for the solubilisation of proteins into reversed micelles. The forward transfer process is extremely fast resulting in the protein in the organic phase in 1 min. The partitioning of lysozyme between an aqueous phase and a reversed micellar phase was found to depend mainly on the electrostatic interactions between the protein and the micellar interface. This major driving force for solubilisation occurred when the charge of the protein was opposite to that of the surfactant. A decrease in solubilisation was observed at low (<3) and high (>12) pH values, where a thick white precipitate was formed at the interface. The precipitate was a complex of denatured lysozyme and AOT. The major factors affecting solubilisation are the protein's pI and the pH and ionic strength of the system. pH values below the pI lead to attractive and therefore favourable interactions with the negatively charged interface of the AOT reversed micelle. It was also shown that the aqueous core of

the micelle could screen the electrostatic interactions between the protein and micelle depending on the ionic strength and salt type of the system. Increases in ionic strength were observed to decrease the solubilisation dramatically.

The surfactant concentration also plays a crucial role on the solubilisation behaviour of lysozyme into AOT/isooctane reversed micelles. Results show that it is the size of the protein molecule relative to the size of the reversed micelle which determines the amount of resistance to solubilisation. Increases in AOT concentration or decreases in the ionic strength lead to larger micelles and to increases in the overall solubilisation. Whereas decreases in AOT concentration and increases in ionic strength lead to smaller micelles and a decrease in solubilisation. It should also be noted that at very low concentrations of AOT there will be insufficient surfactant to solubilise the protein into reversed micelles. At the other end of the spectrum i.e. at very high concentrations of AOT it is unknown whether spherical micelles still exist or whether bicontiuous structures exist.

4.3 Transfer of non-reduced lysozyme into reversed micelles

In the previous section conditions were investigated under which the transfer of native lysozyme partitions into AOT/isooctane reversed micelles. This section reports investigations into the conditions under which the transfer of non-reduced lysozyme transfers into AOT/isooctane micelles. There are no current reports on the transfer of denatured proteins into reversed micelles. This transfer step is crucial if reversed micelles are going to be used for refolding proteins.

Lysozyme was denatured in GuHCl and then transferred into the micelles. The popularity of GuHCl as a protein denaturant rests on the time-tested observation that no other denaturant gives a greater extent of unfolding (Pace 1975, Arakawa and Timasheff, 1984). The forward transfer mechanisms were investigated. An important parameter to be studied was the structural changes in lysozyme that occur in the presence of various denaturants both in and outside the micelles. Fluorescence measurements and CD studies were used to monitor the extent of

denaturation of the protein in these conditions. Unfortunately, no suitable substrate was available to determine the activity of lysozyme inside the micellar environment. The activity was therefore assayed in aqueous solutions before and after the backward transfer steps. The activity results and the structural data enable extent of folding and unfolding of the protein to be determined.

4.3.1 The effect of GuHCl concentration

This section investigates the partitioning of GuHCl denatured lysozyme in the non-reduced state. In the non-reduced state all four disulphide bonds of lysozyme remain intact since no reducing agent is present. The disulphide bonds contribute to the stability of the tertiary structure of the protein. Proteins that are denatured but keep their disulphide bonds intact do not unfold completely to a random coil structure. As the presence of the disulphide bonds holds the protein together preventing full unfolding. The amount of lysozyme transferred using GuHCl denatured lysozyme into 50 mM AOT/isooctane reversed micelles is shown in Figure 4.9. Approximately 90% of the denatured protein is transferred into the micelles between 0.2-1 M GuHCl. Then between 1-1.5 M GuHCl there is a sharp drop in the amount of protein transferred to a value which decreases further to 20% at 2 M GuHCl. Thus the optimum transfer of GuHCl denatured lysozyme occurs at 1 M GuHCl. For comparison, native lysozyme was transferred (10%) at a concentration of 1M KCl in Section 4.2.3.

4.3.1.1 The effect of cations on the size of reversed micelles formed

The effect of various cations on the size of reversed micelles formed was investigated (Figure 4.10). It can be clearly seen that NaCl causes larger micelles to be formed than with KCl or GuHCl. For a salt concentration of 0.5 M, the Wo values for NaCl, KCl and GuHCl are 14.8, 7.7 and 7.0 respectively. As mentioned in the previous section, increased ionic strengths tend to decrease the equilibrium micelle size by compression of the electrical double layer which results in

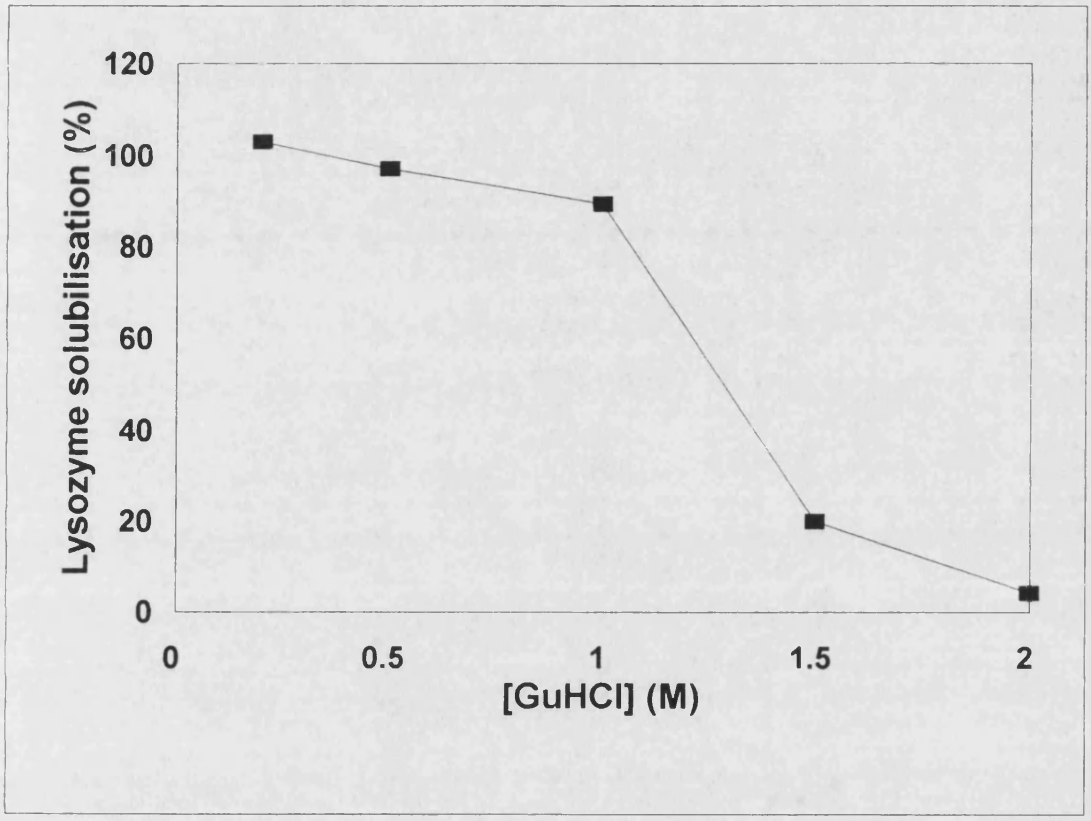

Figure 4.9. The forward transfer of lysozyme (non-reduced) in GuHCl into 50 mM AOT/isooctane reversed micelles

The forward extraction of lysozyme involved mixing equal volumes (5 ml) of 50 mM AOT/isooctane with an aqueous phase (1 mg/ml protein in 0.1 M Tris-HCl buffer containing 0.2-2 M GuHCl) for 15 min. The transfer of lysozyme into the organic phase was recorded (■).

decreased head group repulsions. This can be related to the size of the micelles formed or Wo. Therefore it is not surprising that the sodium ion yields larger micelles than the potassium ion due to the fact that the larger potassium ion binds to the surface of charge more strongly than the smaller sodium ion. The results show that the guanidinium ion forms the smallest micelles and again this can be explained by the larger ion binding strongly to the interface resulting in smaller micelles. As guanidine is a divalent cation whereas sodium and potassium are monovalent cations it would be expected to adsorb more strongly to the micellar interface than the monovalent ions. The results are similar to those obtained by the thermodynamic model of Bruno *et al.* (1990). When the protein is partitioned into the reversed micelle phase it is accompanied by water, potassium and chloride ions whilst the surfactant counterion, sodium, is displaced into the conjugate aqueous phase. Potassium ions can penetrate the surfactant layer more effectively than sodium ions due to their smaller hydrated size, Leodidus and Hatton, (1989). As the aqueous phase ionic strength increases the amount of water taken up into the organic phase decreases due to the reduction in micelle size. For a given salt concentration, Wo increases when the radius of the cation decreases ($Gu^+ > K^+ > Na^+$). Since Na^+ ions are smaller than K^+ ions they neutralize the AOT head groups to a smaller extent than K^+ ions, so effectively the size of the NaCl reversed micelles are larger.

It has been shown that ions that have smaller covalent radii also have a greater degree of hydration associated with them which in turn reduces the propensity to adsorb to surfaces of opposite charge yielding larger micelles. Ions with larger radii have a lower degree of hydration associated with them and can therefore bind more strongly to interfaces of the opposite charge yielding smaller micelles. Increases in ionic strength result in a decrease in equilibrium water solubilisation due to a decrease in micelle size and reduces the transfer of protein into the reversed micelles. Since the guanidinium cations are the largest in size, they bind to the micellar interface to the greatest extent thus forming the smallest micelles.

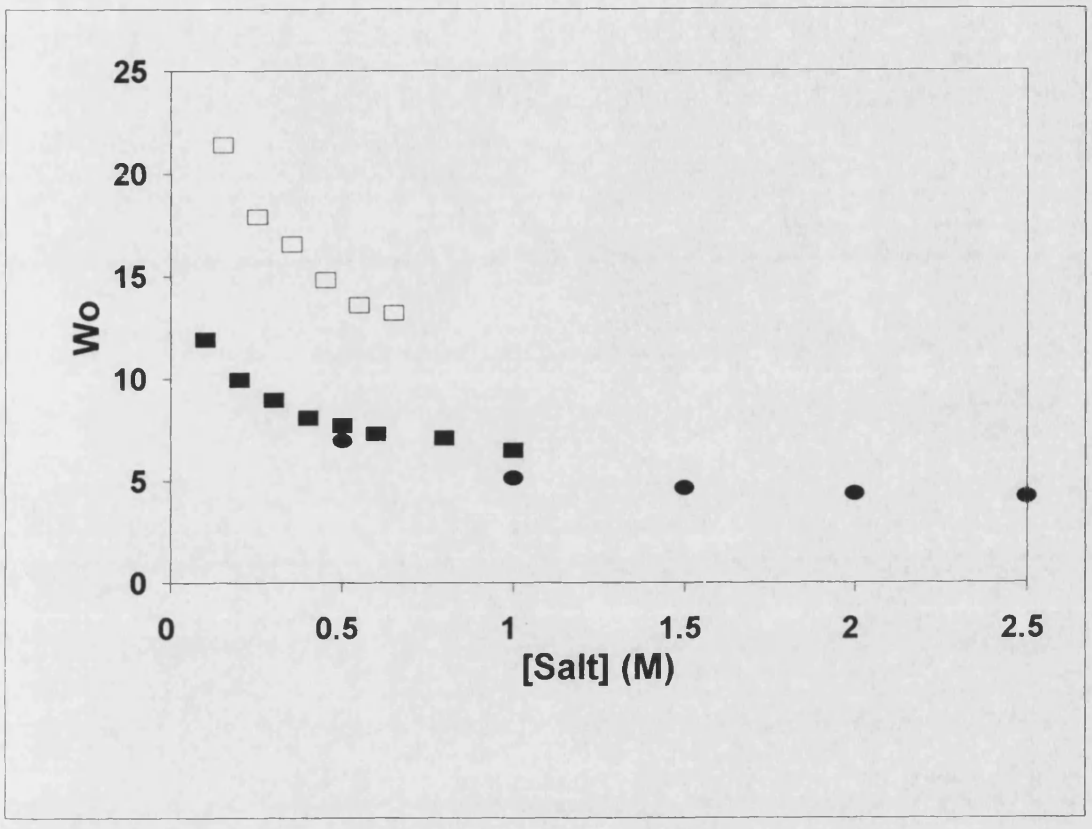

Figure 4.10. The effect of a number of cations on the size of reversed micelles formed

Measurement of water content inside the micelle was studied by mixing equal volumes (10 ml) of 50 mM AOT/isooctane with an electrolyte (□ = NaCl, ■ = KCl and ● = GuHCl) at a particular (0-2.5 M) concentration in 25 mM phosphate buffer, pH 7.0. The samples were mixed for 12 hr to give constant values of water and cations in the organic phase. 0.05 ml of the organic phase was injected into the Karl-Fischer titrator and the amount of water (%) determined. The (%) water was then converted into the Wo parameter.

Results in this section show that GuHCl denatured lysozyme (non-reduced) can be transferred into AOT/isooctane micelles upto a concentration of 1 M. Between 1-2 M GuHCl there is a sharp decline in transfer as seen in Figure 4.9. This can be explained by electrostatic interactions between the positively charged guanidinium ion and the negatively charged AOT head groups. As the GuHCl concentration increases, the repulsive forces between the AOT head groups are reduced and this results in the formation of smaller micelles which expels the protein out from the organic phase. The protein is size excluded from the micelle as the GuHCl concentration increases as shown by the size of micelles formed.

4.3.1.2 Structural studies of non-reduced lysozyme in GuHCl measured by fluorescence

It has already been mentioned that inclusion body proteins require high concentrations of denaturant (6 M GuHCl) to be completely solubilised. In order for this process to be used for refolding, higher denatured protein concentrations must be partitioned into the micelle. Activity measurements of lysozyme denatured in GuHCl between 0.2-2 M showed the protein to have less than 10% of its activity (Section 6.1.1).

Figure 4.11 shows the fluorescence of lysozyme in various concentrations of GuHCl (0-6 M). There is little change in fluorescence between 1-3 M with the relative fluorescence at 350 nm remaining between 120 and 130. However, between 3-6 M a substantial change in the fluorescence intensity at 350 nm is observed with an increase from 130 at a concentration of 3 M to about 200 at 6 M. The increase in fluorescence intensity relates to the unfolding of the lysozyme molecule. This means that between 0-3 M GuHCl no significant changes in structure occur in lysozyme whereas between 3-6 M GuHCl large conformational changes take place causing unfolding of the protein. At 6 M GuHCl lysozyme is completely unfolded and the six tryptophan residues present in the native protein are now exposed to the external environment which relates to the large

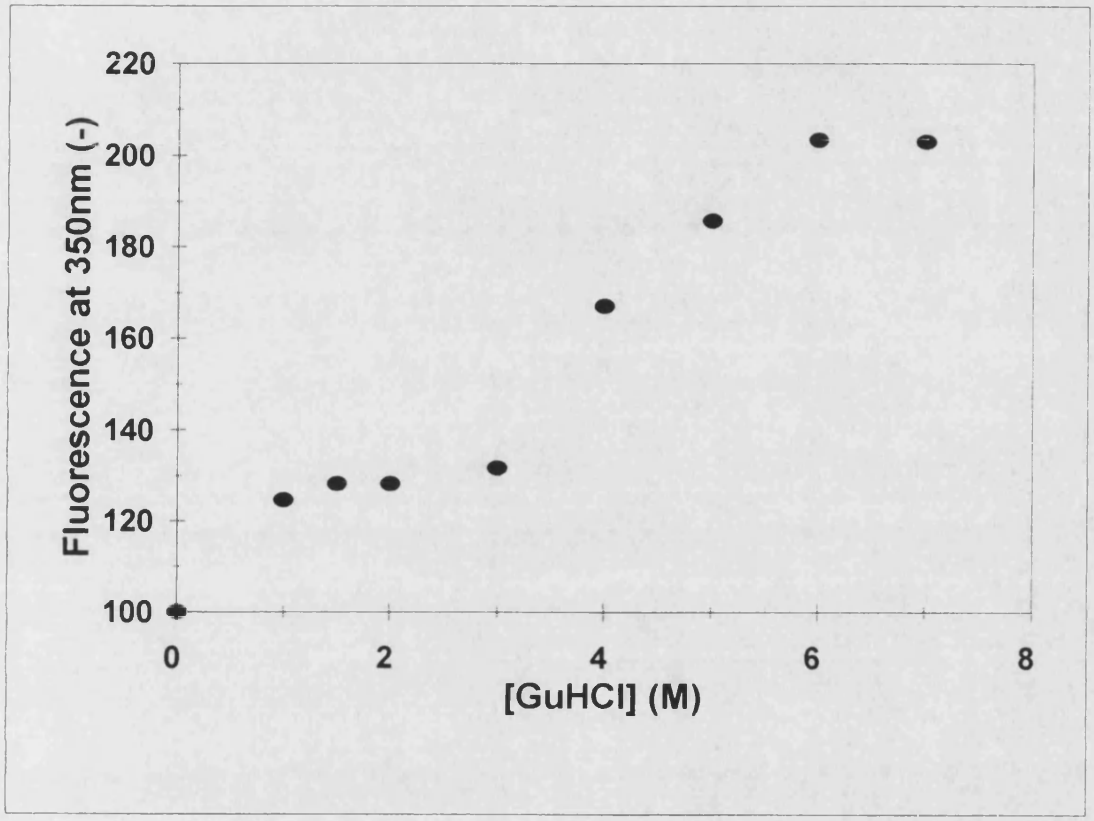

Figure 4.11. Fluorescence of lysozyme in GuHCl

Lysozyme (10 μg/ml) was incubated in GuHCl (●) at concentrations of 1-6 M for 20 min before analysis. The excitation wavelength was 290 nm and the emission spectra were recorded between 300-400. The fluorescence at 350 nm is plotted using arbitary units.

fluorescence value. At 7 M GuHCl no further increase in the fluorescence intensity value is observed indicating that lysozyme is completely unfolded. Steiner (1964) studied the effect of GuHCl concentration on lysozyme by fluorescence and has found that at 6 M the state of the protein is so disorganised that there is little margin for further change.

Results presented previously in Section 4.3.1 showed that lysozyme denatured in GuHCl could only be partitioned into the micelles at concentrations of upto 1 M. The fluorescence data presented in Figure 4.11 proves that very little change in structure is achieved at this low concentration. For any significant unfolding of lysozyme to occur the GuHCl concentration must be between 3-6 M however, transfer into the micelles was found to be impossible at these concentrations (Section 4.3.1). Thus, transfer of lysozyme in 1 M GuHCl places the protein inside the micelle in a near-native form. GuHCl binds to the hydrophilic surfaces of the protein and transfers into the micelle with no unfolding of the protein occurring.

4.3.1.3 Structural studies of non-reduced lysozyme in GuHCl measured by CD in the near-uv region

CD studies were performed in the near-uv where the tertiary structure of the protein could be monitored. Figure 4.12 shows the near-uv CD spectrum of lysozyme in various concentrations of GuHCl. It must be noted that the protein is in the non-reduced form with its disulphide bonds intact. At GuHCl concentrations between 1-3 M, the peak between 280-300 nm does not change significantly indicating that there is no major change in the overall tertiary structure of the protein. This correlates well with the fluorescence data shown previously (Section 4.3.1.2) in that no major structural transitions occurred between 1-3 M GuHCl. However, small changes are occurring in the protein at these concentrations since the native conformation between 280-300 nm has decreased. The band at 260 nm which is characteristic for absorbtion of the disulphide bonds remains the same at these low concentrations since the protein is in the non-reduced form.

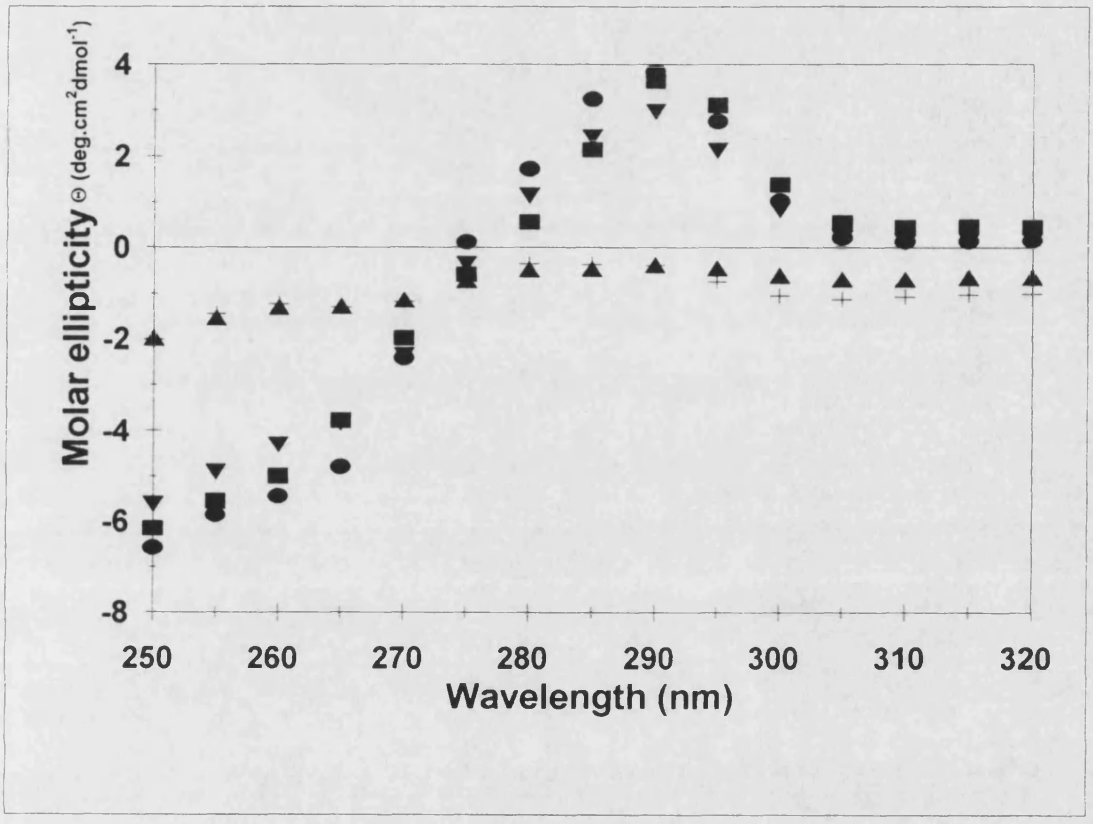

Figure 4.12. Circular dichroism spectra of non-reduced lysozyme (1mg/ml) in GuHCl

Circular dichroism spectra in the near-uv were recorded for lysozyme in GuHCl at various concentrations (■) 1 M, (●) 2 M, (▼) 3 M, (▲) 5 M and (+) 6 M. The samples were left for 30 minutes before the data were recorded between 250-320 nm. The spectra were recorded at a scanning rate of 2 nm/min and are an average of 3 determinations after subtraction of the buffer blank.

At GuHCl concentrations of 5 and 6 M the characteristic peak between 280-300 nm is completely diminished dropping to an ellipticity having value of zero. The spectrum recorded in 6 M GuHCl represents complete denaturation of lysozyme. At this high denaturant concentration lysozyme is in its random coil structure and has completely lost its secondary and tertiary structure. Matsubara *et al.* (1992) have shown that lysozyme in the presence of 6 M GuHCl is in the unfolded state using CD.

The results presented on the tertiary structure of the protein together with fluorescence data in Section 4.3.1.2 confirms the concentration of GuHCl which causes conformational changes to take place is between 3-6 M GuHCl. Both CD and fluorescence data indicate that little change occurs at concentrations of upto 3 M GuHCl. However, between 3 and 6 M GuHCl both sets of data show a change either by an increase in the relative fluorescence or by a decrease in the molar ellipticity value between 280-300 nm which relate to the unfolding of lysozyme. At 6 M GuHCl lysozyme is completely unfolded and has a random coil structure.

The data indicates that GuHCl concentrations must exceed 3 M and preferably be closer to 6 M to completely denature lysozyme. To refold the protein inside the micelle the transfer of the protein into micelles is the next stage to occur. The development of methods to enable partitioning of denatured protein at high GuHCl concentrations now becomes an important step to overcome before refolding inside micelles can take place.

4.3.2 The effect of AOT concentration

Figure 4.13 shows the effect of AOT concentration on the forward transfer of GuHCl-denatured lysozyme into AOT/isooctane reversed micelles. It has been mentioned previously (Section 4.3.1.3) that to completely denature lysozyme a GuHCl concentration of 6 M is needed. In Figure 4.12 it can be seen that at 1.3 M GuHCl only 40% of the lysozyme is transferred into 50 mM micelles whereas 93% is transferred into 400 mM micelles. However, an increase to 2 M GuHCl

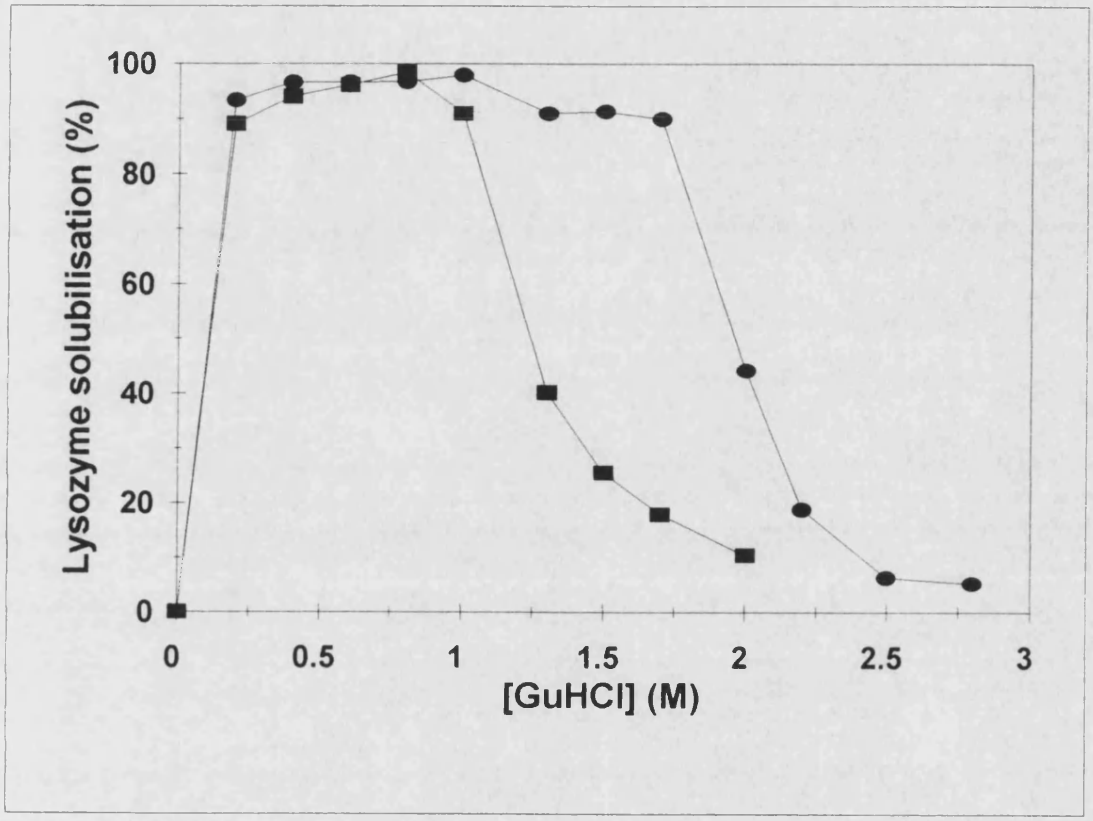

Figure 4.13. The effect of AOT concentration on the forward transfer of denatured, non-reduced lysozyme

The forward extraction of lysozyme involved mixing equal volumes (5 ml) of 50 mM (■) or 400 mM (●) AOT/isooctane with an aqueous phase (1 mg/ml lysozyme denatured at a particular GuHCl concentration in 0.1 M Tris-HCl, pH 8.7) for 10 min. The samples were then centrifuged at 2000 rpm for 10 min and the protein concentration determined in both phases by A_{280} measurements.

resulted in a decline to 40% transfer into the 400 mM micelles. Increasing the AOT concentration leads to increases in micelle size (Göklen 1986) hence an increase in protein transfer will occur. However, increasing the surfactant concentration above 400 mM and up to 1 M (results not shown) showed no further increase in the overall transfer process. Hagen *et al.* (1990) found that the presence of GuHCl has a significant detrimental effect on the transfer process for ribonuclease (RNase) into 400 mM AOT/isooctane micelles. They showed that the transfer of denatured RNase at 0.5 M and 1 M GuHCl gave concentrations of RNase in the organic phase of 0.60 and 0.15 mg/ml respectively. Thus, a four-fold decrease in protein transfer was observed when the concentration of GuHCl was increased twofold. The reason for this result has already been mentioned and is due to the size of the micelles formed decreasing as the denaturant concentration increases (Section 4.3.1.1).

Göklen (1986) has studied the effect of surfactant concentration on the transfer behaviour of a number of proteins including α-chymotrypsinogen, lysozyme, elastase, α-amylase and bovine serum albumin into AOT/isooctane micelles. In all cases there was a much broader solubilisation peak as the AOT concentration was increased. He also showed that more changes occurred when the AOT concentration was increasesd above 50 mM since in this range more significant increases in micelle radius are observed with dynamic light scattering and Karl-Fischer titration. These results confirm that it is the effect of surfactant concentration on micelle size which is the factor responsible for the change in solubilisation behaviour.

The reversed micelles formed at high denaturant concentrations (6 M) are too small to accommodate the protein, thus we encounter the first problem in the overall process. Lysozyme in the experiments to this stage has been in the non-reduced form with its disulphide bonds remaining intact. The results obtained with the reduced form of lysozyme will be presented in the next section.

4.4 Transfer of reduced lysozyme into reversed micelles

This section describes the conditions under which the transfer of the reduced form of lysozyme occurs into reversed micelles. These conditions may or may not be the same as that for the denatured, non-reduced form but it is important to evaluate them. In previous sections data on non-reduced lysozyme has been presented. By reducing lysozyme and breaking the disulphide bonds, the protein is able to form a completely random coil structure. Disulphide bonds play a major role in the stability of proteins. Tamburro *et al.* (1970) studied the role of disulphide bonds in the protein structure of reduced lysozyme and RNase. Their results indicated that there is a large disorganisation of the native molecules when all four of the disulphide bonds are broken. The native state of globular proteins can be thought of as representing a free energy minimum to which the molecule can spontaneously return to from a disordered structure. This minimum energy structure of proteins is mainly determined by intramolecular non-covalent interactions. In addition, the disulphide bonds take up a special position since they exert permanent constraints of covalent character that limits the possible conformations a protein molecule can possess. The reduction of proteins has been discussed in Chapter 3.

4.4.1 The effect of pH

The forward transfer of native lysozyme into micelles was found to be extremely pH dependent and transfer only occurred efficiently at pH values of between 6-10 (Section 4.2.1). At the extremes of pH i.e. pH<3 and pH>13 very little protein transferred into the organic phase and a protein-surfactant complex was formed at the interface.

Figure 4.14 shows the effect of pH on the transfer of reduced lysozyme into AOT/isooctane micelles. The reduction of the disulphide bonds was confirmed by the Ellmans assay before transfer experiments were performed. At low pH (1.5-

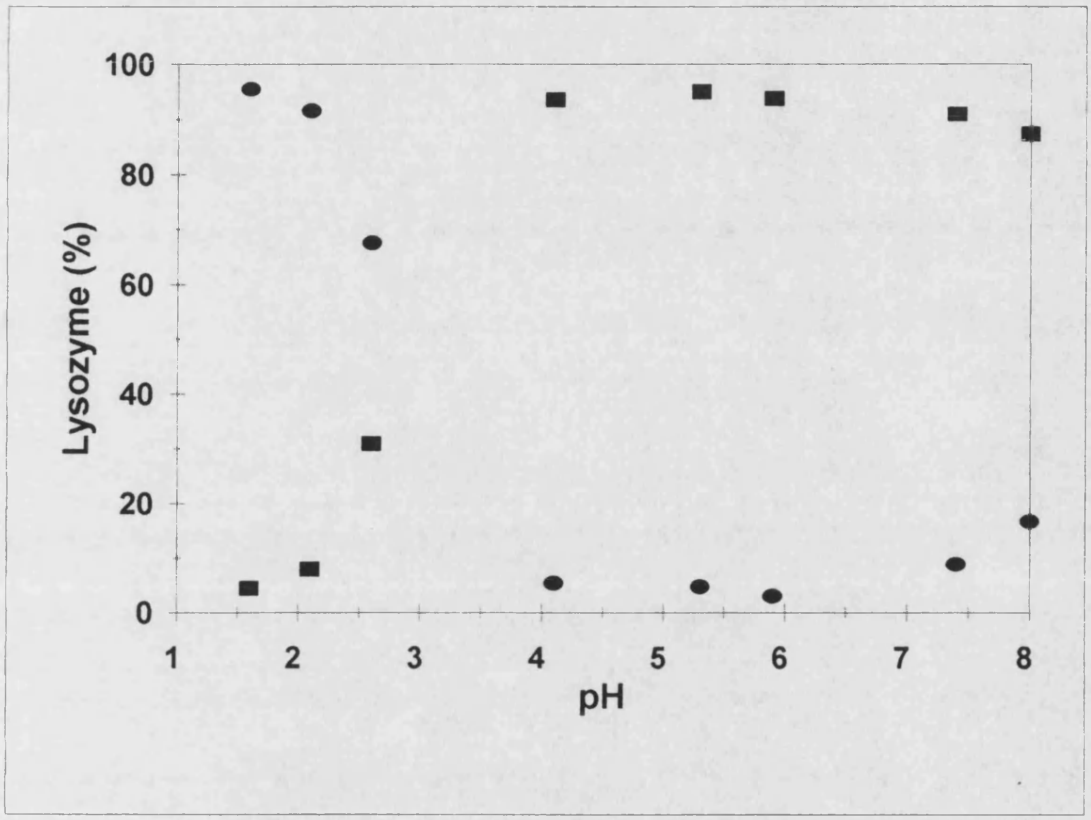

Figure 4.14. The effect of pH on the forward transfer reduced lysozyme in 2 M GuHCl into 50 mM AOT/isooctane micelles

The forward extraction of the protein involved mixing equal volumes (5 ml) of 50 mM AOT/isooctane with an aqueous phase (1 mg/ml reduced lysozyme in 25 mM phosphate buffer at a particular pH (1.5-8.0) for 15 min. The phases were then centrifuged at 2000 rpm for 10 min to give two clear phases. The protein concentration in the organic (●) and aqueous (■) phases was determined.

2.2) the protein can be transferred into the organic phase to between 90 and 100% and as the pH increases to pH 2.6 transfer declines to 70% (Figure 4.14). Between pH 4-7.4 very little transfer occurs with less than 10% of the protein detected in the micellar phase. At pH 8 the protein transfer increases slightly to 20%. The transfer of reduced lysozyme above pH 8 were not peformed since the reduced protein may become oxidized in the alkali pH values. Figure 4.14 also shows the corresponding protein remaining in the aqueous phase with the mass balance between the two phases showing an error of less than 5%.

The behaviour of reduced lysozyme is the reverse of the transfer of native lysozyme under the same conditions. It was shown previously (Section 4.2.1) that very little native protein could be partitioned into micelles at low pH and a large amount of precipitate was visually observed. Native protein was transferred (90%) at the intermediate pH values between 6-10. In this instance the protein is in its reduced state and there exists the possibility of more interaction between the guanidinium ions and the protein. As the reduction of the protein enables it to from a slightly less native structure making additional binding sites on the protein available for guanidinium ions to interact with. The negatively charged surfactant heads may also interact in some way with the protein.

The mechanism for the forward transfer of reduced lysozyme is not as straight forward as that of native protein. Electrostatic interactions do not play any part in the transfer process since the best transfer occurs at low pH (1.5-2.3). At these low pH values lysozyme is far from its pI (10.9) and consequently it contains many negative charges. If electrostatic interactions played a part in this transfer then the protein would be repelled out of the micellar core resulting in poor transfer. A possible explanation for the transfer is that at the low pH values the protein carries a high amount of negative charge which allows the positively charged guanidinium ions to bind onto the protein. The guanidinium ions are more extensively bound to the protein and this charge is now attracted inside the micelle. However, there must be a limit for the reduced protein in GuHCl to be

transferred as the positive charge will reduce the repulsion between AOT head groups causing the micelle to shrink.

The reason for the poor partition of the reduced protein at intermediate pH values (4.1-8) is unknown. The only comparison that can be made is with similar studies on RNase by Hagen (1989) who found no difference in the transfer between reduced and native RNase as a function of pH into 400 mM AOT/isooctane micelles.

In summary, the transfer of reduced lysozyme into reversed micelles behaves differently to the non-reduced form and the native form. Transfer occurs at very low pH (1.6-2.5) with a sharp decline as pH increases to intermediate values (4.1-8.0). This process does not obey the electrostatic interaction theory as described in Section 4.2.1. The transfer that occurs must be a result of the increased interactions between the guanidinium ion and the protein, which in turn is attracted into the interior of the micelles due to the anionic head groups. However, there must be an upper concentration limit to the amount of reduced lysozyme in GuHCl into reversed micelles. This will be presented in the next section.

4.4.2 The forward transfer of reduced lysozyme as a function of GuHCl concentration.

It has previously been shown that the optimum concentration for the transfer of non-reduced lysozyme into micelles is 1 M GuHCl (Section 4.3.1). In the previous section reduced lysozyme was successfully partitioned into micelles at a concentration of 2 M GuHCl but it was found to be pH dependent. Figure 4.15 shows the transfer of reduced lysozyme as a function of GuHCl concentration. At GuHCl concentrations of 0.2-3 M between 90-100% of the protein is transferred into the organic phase. The reduced form of the protein, which has had the disulphide bonds broken, allows the protein to assume a slightly less native structure. Figure 4.15 shows that even at concentrations of 3 M GuHCl most of the reduced protein is transferred into the micelle. However, at a concentration of

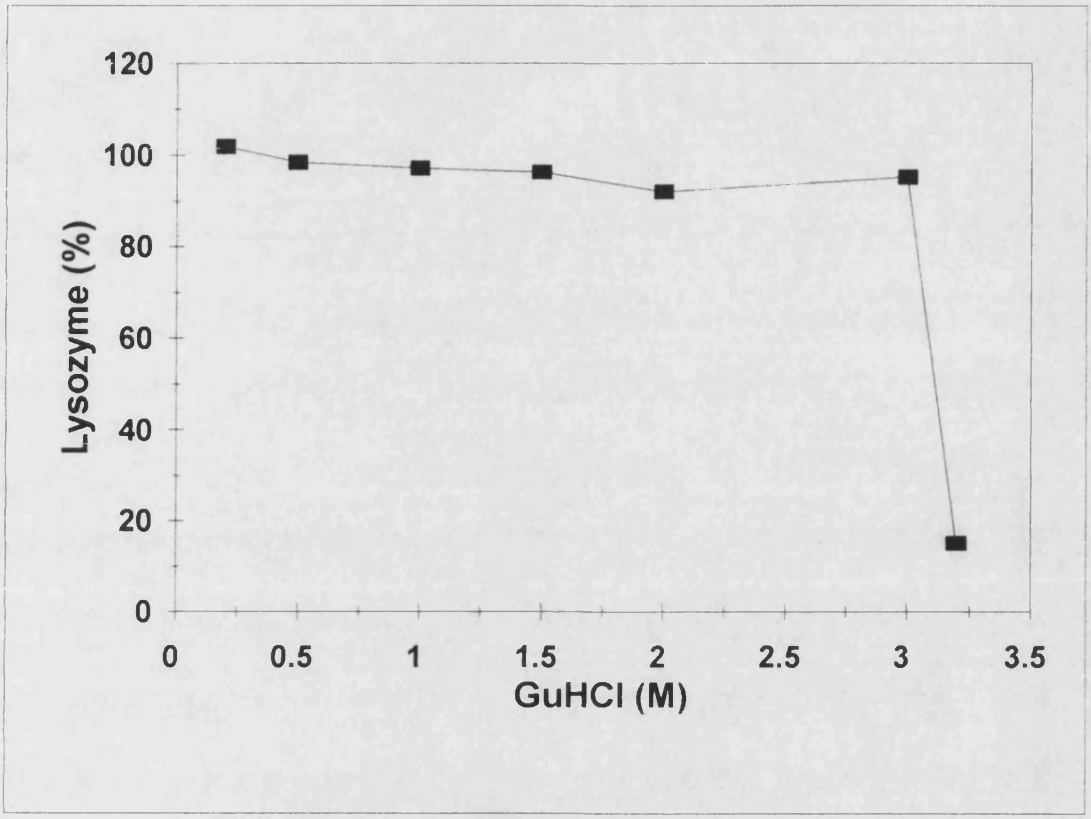

Figure 4.15. The forward transfer of reduced lysozyme as a function of GuHCl concentration into 50 mM AOT/isooctane micelles

The forward transfer of the protein involved mixing equal volumes (5 ml) of 50 mM AOT/isooctane with an aqueous phase (1 mg/ml reduced lysozyme in 25 mM phosphate buffer, pH 2.0) for 15 min. The phases were then centrifuged at 2000 rpm for 10 min to give two clear phases. Protein concentration was determined in the organic phase (■).

3.2 M GuHCl a sharp drop in the transfer occurs yielding less than 20% of the protein in the micelle.Thus, there is a significant difference in the transfer of reduced and non-reduced protein. As explained previously, the non-reduced form of lysozyme with its 4 disulphide bonds intact, is probably in a native-like form when in a 1 M GuHCl solution. This concentration of GuHCl is not strong enough to unfold the protein molecule so the structure is still globular. The transfer of the globular proteins into reversed micelles is thought to occur by the deformation of the interfcace around the protein followed by pinching off the surfactant layer around the protein. The process has been described by Göklen (1986) and involves a protein diffusing towards the aqueous-organic interface where there is a monolayer of surfactant. It then forms a micelle around itself by carrying water and surfactant with it into the organic phase. When the protein is in its reduced nearly native structure the transfer must occur by a different process since it can be transferred into micelles at GuHCl concentrations that is not possible for the non-reduced protein. In the nearly native conformation the positively charged guanidinium ions are bound to the protein molecule and therefore a surplus of positive charges approach the negative charged surfactant layer. This surplus of bound positive charges to the protein is attracted towards the negatively charged interface.

Hagen (1989) has postulated that this increased interaction may facilitate the transfer of denatured RNase into the reversed micelles far more than the native protein. There may have been favourable interactions between hydrophobic residues exposed in the unfolded molecule and the surfactant tail regions.

The explanation for the transfer of RNase can probably be assumed for lysozyme. Guanidinium ions bind to all parts of the open molecule and the interaction between the surfactant head groups and the guanidinium ion facilitates the transfer of protein at higher denaturant concentrations into the micelles.

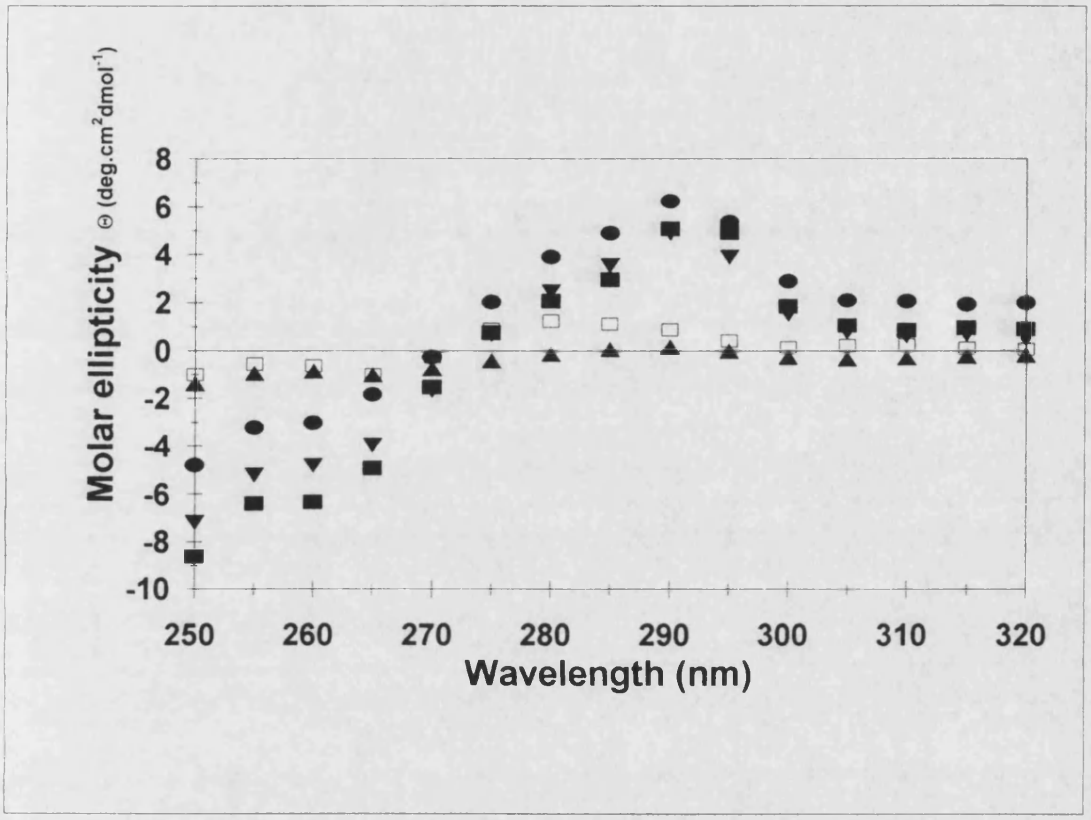

Figure 4.16. Near-uv circular dichroism spectra of reduced lysozyme (1 mg/ml) in GuHCl

Near-uv Circular dichroism spectra were recorded for reduced lysozyme in GuHCl at various concentrations, (■) 1 M, (▼) 2 M, (●) 3 M, (□) 4 M and (▲) 6 M. The samples were left for 30 minutes before data were recorded between 250-320 nm. The spectra were recorded at a scanning rate of 2 nm/min and are an average of 3 determinations after subtraction of the buffer blank.

4.4.3 The effect of GuHCl concentration on the structure of lysozyme measured by CD in the near-uv region

The tertiary structure of lysozyme has been investigated by near-uv CD for the reduced form of lysozyme in various concentrations of GuHCl (Figure 4.16). The reduced form of the protein, with its disulphide bonds broken in GuHCl, should give different spectra than the non-reduced form. Between 1 and 3 M GuHCl most of the tertiary structure of the protein is retained as there is little change in the spectra in the band between 280-300 nm. It should be noted that the band is different to the one present in the non-reduced form with GuHCl (Figure 4.12). For the non-reduced form of lysozyme, the molar ellipticity at 290 nm is 6000 in 2 M GuHCl compared to a value of about 5000 for the reduced form of the protein at the same denaturant concentration. Breaking the disulphide causes slight change in the tertiary structure of lysozyme at these low denaturant concentrations. The Figure shows that at a concentration of 4 M GuHCl the peak at 290 nm has nearly diminished revealing that almost all of the tertiary structure of lysozyme in the reduced form is lost. The denaturant concentration required for complete loss in tertiary structure is lower (4 M) than that of the non-reduced form, which requires 6 M GuHCl as shown in Figure 4.12. The band at 260 nm also decreases with increasing GuHCl concentrations and this relates to the reduction of the protein allowing a more of a disorganised structure to exist.

Results presented here indicate that at least a concentration of 4 M GuHCl is required to completely denature lysozyme in the reduced form. Concentrations of GuHCl below 4 M do not unfold lysozyme to any significant degree and the protein exists in a nearly native state.

Results presented in Section 4.4.2 showed that reduced lysozyme in GuHCl could be partitioned at concentrations of up to 3 M. The CD studies indicate that at 3 M GuHCl lysozyme retains most of its tertiary structure. However, at 4 M GuHCl the peak at 290 nm is almost diminished to zero revealing that very little tertiary structure remains. Reduced lysozyme cannot be partitioned into micelles at 4 M

GuHCl and can only be transferred at concentrations of up to 3 M GuHCl, therefore lysozyme is transferred in the nearly native state. In addition as these GuHCl concentrations are likely to be too low to dissolve inclusion bodies, an alternative method must be employed to enable the transfer of denatured lysozyme into micelles.

4.5 The use of urea as a denaturant

The partitioning of denatured lysozyme into AOT reversed micelles using GuHCl as the denaturant does not occur at the high concentrations required. This is due to the charge interacations between the positive guanidinium ion and the negative surfactant head groups which cause the micelle to shrink and expell the protein. Therefore, it is necessary to use an alternative denaturant to allow partitioning of the denatured lysozyme. Urea is another chaotrope commonly used to denature and unfold proteins (Pace, 1975 and Creighton, 1990). Generally, higher concentrations of urea are required to cause complete unfolding of a protein compared to GuHCl but the denatured states are similar (Pace, 1975). The forward transfer of lysozyme in the presence of GuHCl into AOT/isooctane was limited to a concentration of 1.0 M for non-reduced lysozyme and 3 M for reduced lysozyme. Both of these concentrations of GuHCl were not high enough to unfold the protein molecule as revealed by CD data.

Experiments were performed to establish whether urea could be used as an alternative denaturant to GuHCl and applied to this system. The possible advantage urea may have in this system compared to GuHCl is that there is no charge associated with it. Thus, if urea denatured lysozyme could be partitioned into reversed micelles there will be no charge problem associated with the negatively charged surfactant head groups and the neutral urea molecule. Therefore, the micelles would not shrink in size as they do with GuHCl due to the charge interactions and it may be possible to completely unfold lysozyme and transfer it into the micelles.

Table 4.1. The forward transfer of lysozyme in urea into 50 mM AOT/isooctane reversed micelles.

The forward transfer of lysozyme involved mixing 5 ml of an aqueous phase (1 mg/ml lysozyme in urea (1-8 M) in 25 mM phosphate buffer, pH 7 and 0.1 M KCl) with 5 ml of an organic phase (50 mM AOT/isooctane). The results of both the organic and aqueous phases were noted.

Urea (M)	Organic Phase	Aqueous Phase
1	cloudy thick white ppt org phase >> aq phase	v.small clear phase
2	cloudy thick white ppt org phase >> aq phase	v.small clear phase
3	cloudy thick white ppt org phase >> aq phase	clear phase
4	cloudy thick white ppt org phase = aq phase	clear phase
6	completely clear gel	1 phase
7	cloudy thick white ppt org phase < aq phase	clear phase
8	cloudy thick white ppt org phase << aq phase	clear phase

Lysozyme (1 mg/ml) was solubilised in increasing concentrations of urea (1-8 M) and 0.1 M KCl, then the phase transfer method was employed to transfer the protein into 50 mM AOT/isooctane micelles. A mixing time of 15 minutes was used. Table 4.1 shows the observations on the forward transfer of lysozyme in urea using the phase transfer method. It was found to be impossible to detect the protein in either the aqueous or organic phase. In all the samples equal volumes (5 ml) of both phases were mixed together. However, after leaving the solutions to phase separate in a water bath at 25°C the two phases did not contain equal volumes. The relative size of the 2 phases is indicated in Table 4.1. Another observation made was that the phases were of a viscous nature and appeared to be gel-like. The aqueous phase was always clear but the protein concentration could not be determined due to the gel-like composition of the phase. The organic phase on the other hand was in the form of a milky precipitate. The other interesting point was that as the denaturant concentration increased from 1-8 M the size of the organic phase became smaller and the aqueous phase became larger. Table 4.1 shows that between 1-3 M the organic phase was larger than the aqueous phase and between 7- 8 M the situation was reversed. At 6 M the original two phase system had been transformed into a clear 1 phase gel.

The reason for the formation of these gels is unclear and at present there is no explanation for this behaviour. It was thought that perhaps the alkane (isooctane) phase may have not been compatible with the system so a number of different alkanes were used ranging from hexane to hexadecane but no difference was found using other alkanes. Experiments were also performed with and without the presence of protein, with varying amounts of KCl (0.1-1 M) and different times for forward extractions were used (1-120 min) and also 50-400 mM AOT/isooctane, but the resulting gel-phase always occurred.

Hagen (1989) found by mixing equal volumes (5 ml) of 400 mM AOT/isooctane with an aqueous solution of urea in 25 mM phosphate buffer, pH 7.5 for 15 min that the water content increased up to a Wo value of 50 at 4 M. It was postulated that urea may have been acting as a cosurfactant, intercalating between the AOT

molecules, thus increasing the size of the micelles and hence the water content. Hagen (1989) also observed that at the highest Wo, the appearance of the solutions started to change and other surfactant configurations such as lamellar structures may have been present.

The experiments performed were repeated with urea exactly as stated by Hagen (1989) however it was not possible to obtain similar results. A possible explanation may have been due to the different supplies of AOT. AOT was purchased from Sigma (99.9%) whereas Hagen purchased AOT from Pfalz and Bauer. Luisi and Steinmann (1987) have shown that different batches of AOT contain certain impurities which can give different results in this field of work. They state that there are a number of different commercial sources available for the purchase of AOT. For the majority of technical applications the check of chemical purity of the surfactant is not essential but in work with relevance to enzyme activity and spectroscopy it is possible that a number of artifacts may originate form the surfactant. Luisi and Steinmann (1987) have found that the majority of commercial supplies of AOT are contaminated with UV-absorbing impurities and acid impurities as well as salts. After discovering these impurities in the surfactant, Luisi used a HPLC purification technique which eliminated any UV-absorbing acid impurities from the commercial samples. It should be noted that the AOT used within this thesis was purchased from Sigma and was of high purity. Furthermore, surfactant impurity is not the major problem that it was several years ago.

Although urea has no charge associated with it the partitioning of lysozyme into the aqueous core of the micelle using the phase transfer method was unsuccessful. The resulting two phases after separation were of a gel-like structure, unequal in volume with both the organic and aqueous phase compositions very different. Protein concentration could not be determined due to this unusual gel-structure. It is obvious that as the urea concentration is increased some kind of transition occurs as both the volumes and contents of the phases changed. An explanation

might be that urea has a different solubility limit in the small volume of water present inside the micelles compared to its limit in an aqueous solution.

4.5.1 The effect of urea on the structure of non-reduced lysozyme measured by fluorescence

Section 4.5 examined the partitioning of denatured lysozyme in urea into micelles. However, under the conditions employed no transfer was achieved. Fluorescence was used to monitor the changes in structure of lysozyme when incubated in increasing concentrations of GuHCl (Section 4.3.1.2). The effect of urea on lysozyme structure was also monitored by fluorescence.

The unfolding of lysozyme in urea does not occur to the same extent as when GuHCl is used as a denaturant (Figure 4.17). Between 1-8 M urea the fluorescence at 350 nm rises from 100 to about 130. Thus, even at a concentration of 8 M urea there seems to be very little change in the structure of the protein. The structural changes that occur in 8 M urea are the same as those which occur in 3 M GuHCl.

GuHCl is believed to affect both the hydrophobic and ionic interactions in the protein, whereas urea only affects the hydrophobic interactions. The CD of lysozyme in the presence of GuHCl and urea has been studied by Matsubara *et al.* (1992). They found that lysozyme was completely unfolded in 6 M GuHCl but in 8 M urea the spectra was the same as that for the native protein and only a very slight change was observed in 10 M urea. The CD observations monitor secondary structure changes in the protein and confirms the data obtained by fluorescence. The results presented indicate that urea is inefficient for unfolding lysozyme and it is a weak chaotropic reagent in comparison with GuHCl. Urea only unfolds lysozyme via hydrophobic interactions since it posesses no charge. In this way changes in structure are small since ionic interactions are also required to unfold proteins. This is probably due to the fact that there are no ionic interactions between urea and lysozyme and the hydrophobic interactions are not sufficient enough to unfold the molecule.

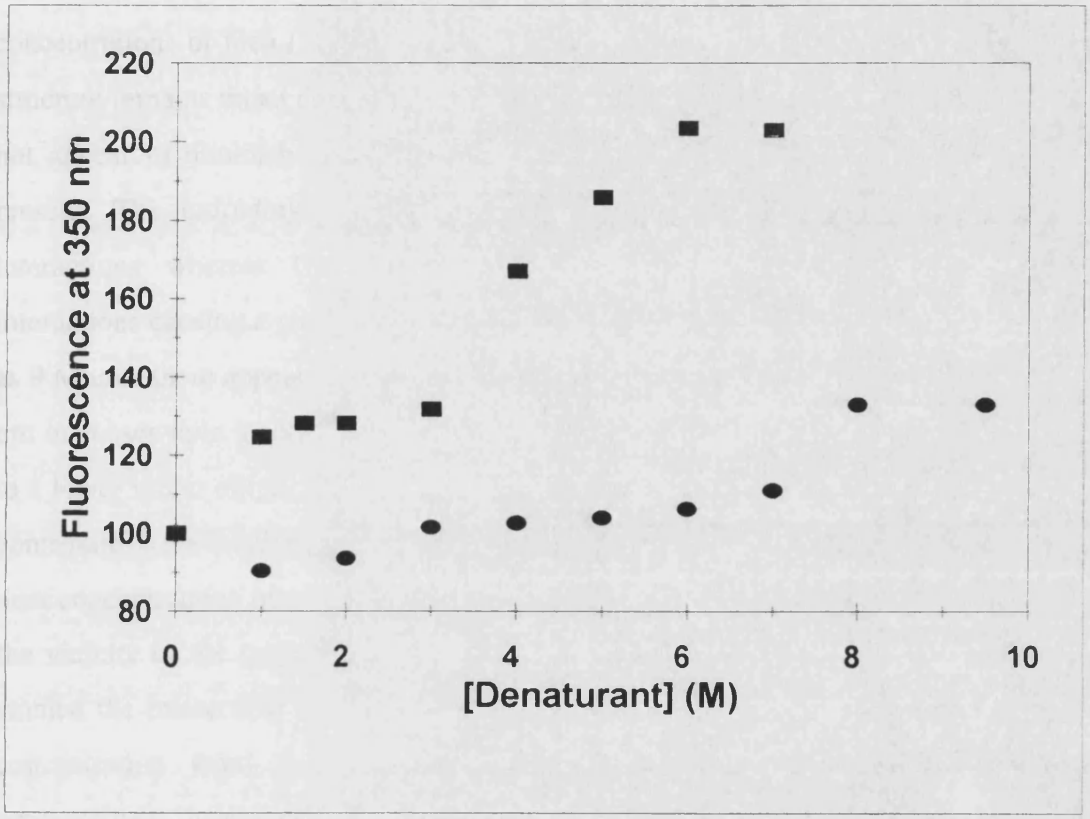

Figure 4.17. Fluorescence of lysozyme in urea and GuHCl

Lysozyme (10µg/ml) was incubated in GuHCl (■) 1-8 M and urea (●) 1-9.5 M for 20 mins. Samples (1 ml) were excited at a wavelength of 290 nm and the emission spectra were recorded between 300-400 nm. The relative fluorescence is plotted using arbitary units.

4.5.2 The effect of urea on the structure of non-reduced lysozyme measured by CD in the near-uv region

The tertiary structure of non-reduced lysozyme in urea was investigated by near-uv circular dichroism. Near-uv spectra of lysozyme were measured in increasing concentrations of urea (1-9 M) and is shown in Figure 4.18. Most of the tertiary structure remains intact even at concentrations of 9 M as the band at 290 nm does not appear to diminish so it appears that most of the tertiary structure is still present. The unfolding of proteins with urea only affects the hydrophobic interactions whereas GuHCl can affect both the ionic and hydrophobic interactions causing a greater unfolding. Therefore, at concentrations even as high as 9 M urea there appears to be very little unfolding of lysozyme. The band at 290 nm increases upto a concentration of 9 M urea but at 10 M the peak is decreased to a lower molar ellipticity value. It is the tryptophan and tyrosine residues which contribute to the band at 290 nm and this enhancement in the band with increasing urea concentrations must relate to some disruption of the protein conformation in the vicinity of the tyrosine and tryptophan residues. Warren and Gordon (1970) studied the interaction of urea with lysozyme and found that increasing the urea concentration from 3-9 M the interactions between lysozyme and urea progressively increased but no significant changes in the optical rotatory parameters or intrinsic viscosity of the protein occurred. Therefore, the disruption of lysozyme conformation in the presence of 9 M urea was small. However, only small changes are observed in the structure of lysozyme as some tertiary structure remains, even in 10 M urea, since the peak at 290 nm is still apparent.The results for urea differ from those obtained with GuHCl as when the GuHCl concentration is increased, the peak at 290 nm decreases.

The results presented in Figure 4.18 are similar to the results presented by Barnes *et al.* (1972). Barnes *et al.* (1972) studied the effect of urea on lysozyme as measured by CD. They found that concentrated solutions of urea did not induce any extensive disruption of the native conformation of lysozyme. The addition of urea to the lysozyme solution resulted in the positive band at 290 nm and the

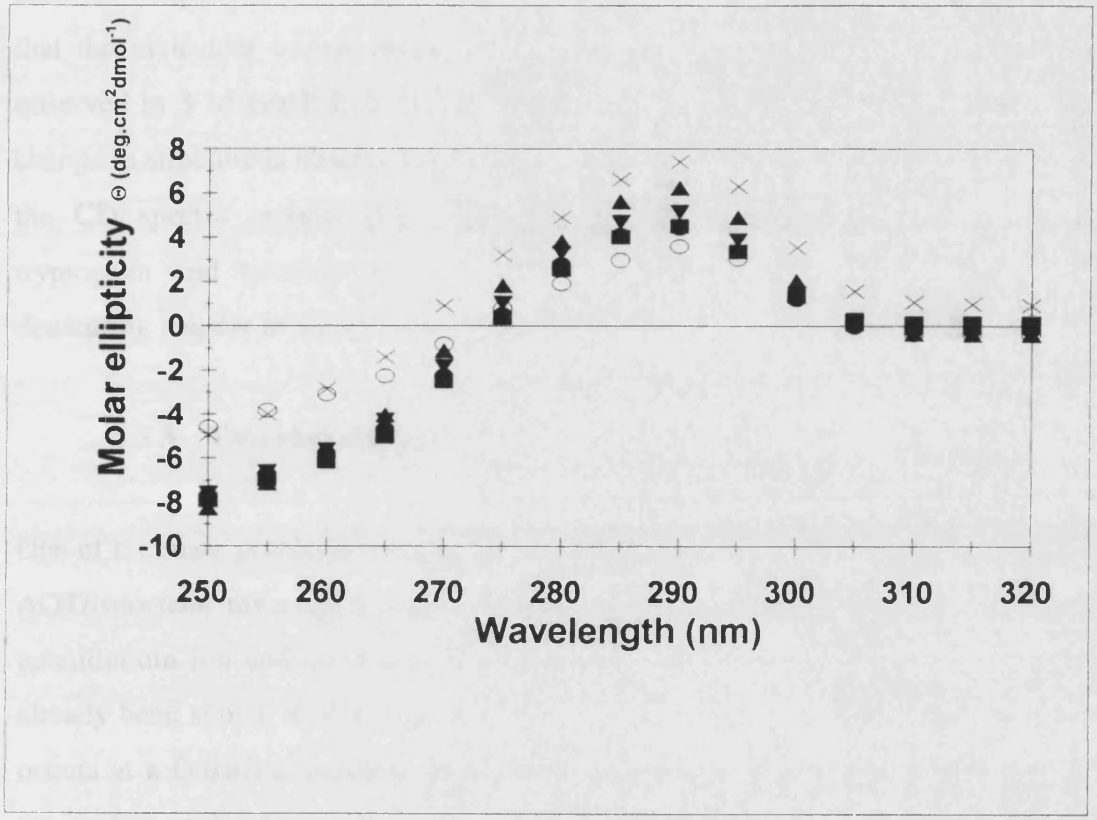

Figure 4.18. Near-uv Circular dichroism spectra of non-reduced lysozyme (1 mg/ml) in urea

Circular dichroism spectra in the near uv were recorded for lysozyme denatured in urea at various concentrations, (■) 1 M, (●) 2 M, (▼) 3 M, (▲) 7 M, (×) 9 M and (○) 10 M. The samples were left for 30 minutes before data were recorded between 250-320 nm. The spectra were recorded at a scanning rate of 2 nm/min and are an average of 3 determinations after subtraction of the buffer blank.

negative plateau around 258 nm to increase as the concentration of urea was increased. Steiner (1964) has studied the structural transitions of lysozyme in GuHCl and urea. He found that some residual structure survived the denaturing effects of 9 M urea. This residual structure could be lost at extremes of pH, elevated temperatures, or by the reduction of the disulphide bonds. Steiner states that the molecular events occurring in 9 M urea are similar to the transitions observed in 3 M GuHCl. Both the fluorescence and CD data confirm that little change in structure is observed in the presence of urea. Slight changes are found in the CD spectra around 290 nm showing that the environment around the tryptophan and tyrosine residues is altered. In conclusion, urea is a poor denaturing reagent in comparison with GuHCl for unfolding lysozyme.

4.5.3 Two step dilution process for refolding

One of the main problems associated with the transfer of denatured lysozyme into AOT/isooctane reversed micelles is the charge between the positively charged guanidinium ion and the negative charges on the surfactant head groups. It has already been shown that the optimum transfer of lysozyme into reversed micelles occurs at a GuHCl concentration of 1.5 M (Section 4.3.1). At this concentration the protein molecule remains folded and is in the near-native state as shown by fluorescence data (Section 4.3.1.2).

The partitioning of protein at a concentration of 6 M GuHCl into reversed micelles is not possible therefore a two stage dilution could be usefully employed. The protein is completely denatured in 6 M GuHCl after which this solution is diluted to a denturantat concentration which allows maximum transfer of the protein into the reversed micelles.

CD can be used to measure the tertiary structure of a given protein in order to establish the extent of denaturation given by a specific chaotrope. The near-uv CD spectrum of lysozyme is shown in the presence of 6 M GuHCl after which it was diluted to 2 M and 3 M respectively (Figure 4.19). When the protein is reduced

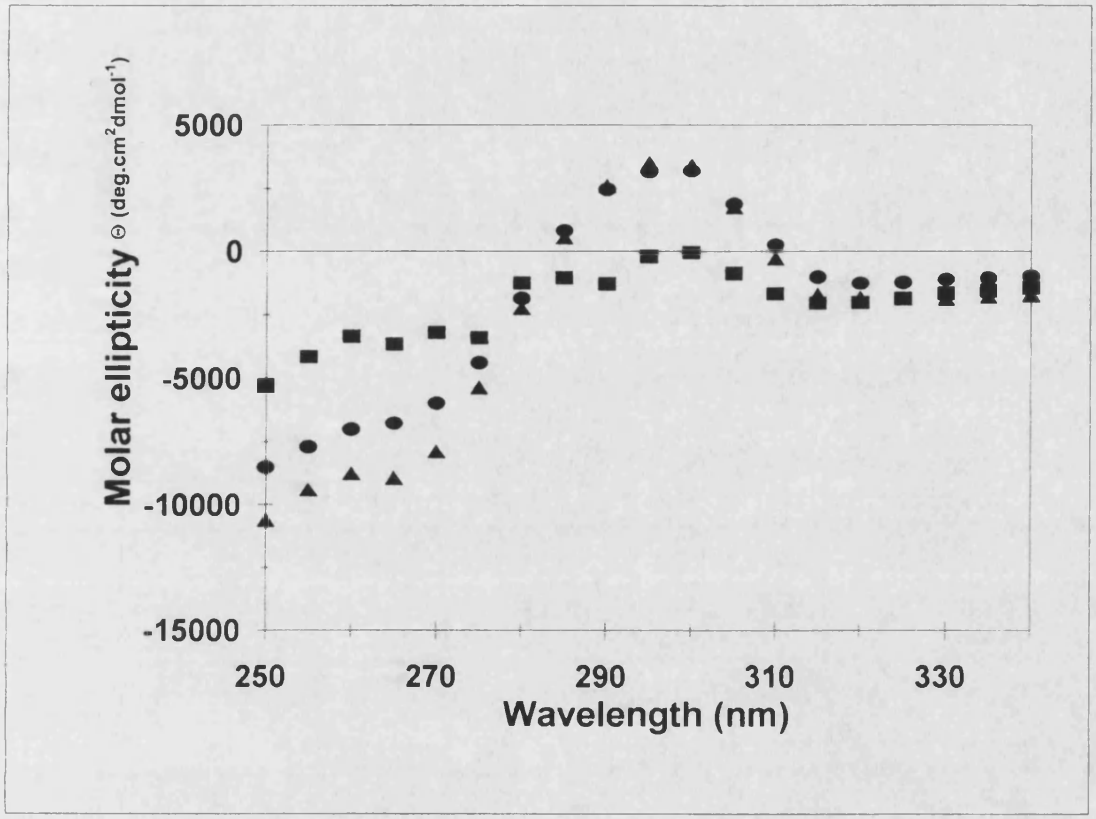

Figure 4.19. Circular dichroism spectra of reduced and denatured lysozyme after dilution

Circular dichroism spectra were recorded in the near u.v. for reduced and denatured lysozyme (1 mg/ml) in 6 M GuHCl (■). This was then diluted to 3 M (▲) and 2 M (●) respectively. The samples were left for 30 minutes after which spectra were recorded between 250-320 nm. A scanning rate of 2 nm/min was used and an average of 3 determinations were made.

and denatured in 6 M GuHCl the molar ellipticity is negative and the peak that normally occurs between 280-320 nm corresponding to tyrosine and tryptophan residues disappears showing a loss of tertiary structure. Diluting the sample to 3 M and 2 M GuHCl respectively results in the peak at 280-320 nm reappearing and this indicates that some of the tertiary structure can be regained by a 2-3 fold dilution. The dilution step also reduces the concentration of the reducing agent and this allows the protein to recover some of its native characteristics. Another characteristic observed on the graph is the decrease in molar ellipticity occurring at between 250-260 nm for each of the solutions. This can be attributed to the disulphide bonds.

A two fold dilution process has been used with RNase (Hagen, 1989). The protein was denatured and reduced in 6 M GuHCl then diluted to 1 M and transferred into micelles. No data was presented on the structure of the protein after this dilution step. However, it was verified that the disulphide bonds were still in the reduced form. It is possible that in the work of Hagen (1989) that RNase was being transferred into AOT/isooctane reversed micelles in the partially denatured state.

The other possibility of using a two step dilution process was investigated. In this process the protein was completely denatured in 6 M. This solution was then diluted to 2 M or 3 M GuHCl and transferred into the reversed micelles. However, CD data showed that these dilutions resulted in some of the tertiary structure returning as monitored by the peak between 280-300 nm. This meant that if the protein was transferred under these conditions, the protein would have been transferred in a partially denatured state. The two stage dilution was discarded as a possibility after this stage.

4.5.4 Summary

The partitioning of non-reduced and reduced lysozyme into AOT/isooctane reversed micelles has been investigated. The conditions required to transfer the

non-reduced and reduced form of the protein are very different to that for native lysozyme. Native lysozyme transfers into micelles mainly due to electrostatic interactions between the protein and the surfactant's interface. These interactions do not take part on partitioning of the non-reduced and reduced form of the protein into micelles.

Transfer of non-reduced lysozyme occurs in a similar fashion to native protein with the maximum transfer occurring at a concentration of 1 M GuHCl. The protein is nearer to the native conformation than the unfolded one. Lysozyme possesses it's globular structure because the four disulphide bonds present in the protein restrict the protein from having a random coil structure. Fluorescence studies showed that no major change in the structure of lysozyme occurred below concentration of 3 M GuHCl and between 3-6 M GuHCl lysozyme was unfolded. These results were confirmed by the CD and the fluorescence which showed that major transitions in structure occurred after 3 M and at 6 M unfolding was complete. This data showed that a concentration between 3-6 M GuHCl is required, preferably 6 M, to completely unfold lysozyme and then denatured lysozyme can be transferred into AOT reversed micelles. Once denatured lysozyme can be transferred, refolding experiments can proceed.

The transfer of reduced lysozyme into AOT micelles was investigated. With the disulphide bonds broken the protein is no longer constrained by its disulphide bonds and is allowed to adapt a more randomly coiled structure. The transfer process was found to be very pH dependent and transfer only took place in acidic conditions. This can not be explained by electrostatic interactions between the protein and micelles interface. At the low pH values lysozyme possesses a significant amount of negative charge which would be repelled by the micellar interface. However, reduced lysozyme is found to be attracted into the micelles. The data also shows that higher concentrations of reduced lysozyme can be partitioned into the micelles than the non-reduced form of the protein. This can be explained by the fact that reduced protein has a more disorganised structure which provides additional binding sites for the guanidinium ion. Since more guanidinium

ions can bind to the potein it is attracted into the micelle due to the opposite charge. However, there is a limit to the transfer of the protein due to the GuHCl concentration. CD studies show that the tertiary structure of the reduced form of lysozyme is completely lost at a concentration of 4 M GuHCl. The denaturant concentration at which transfer can occur is still not high enough to transfer completely unfolded lysozyme into the reversed micelles.

Attempts were made to transfer lysozyme in urea into AOT micelles using the phase transfer method. On contacting the aqueous phase with the organic phase a gel-like structure was formed. Protein concentrations could not be detremined due to the viscous nature of the phases. The reason for this is unclear but a possible explanation may be that the solubility limit of urea inside the micelles might be different to that in an aqueous phase. Fluorescence and CD data revealed that very little change in the tertiary structure of lysozyme occurred at concentrations of upto 10 M urea.

4.6 Summary of Chapter 4

This section provides a summary for the forward transfer of native and non-native lysozyme into AOT/isooctane reversed micelles:

- Initially phase diagrams were constructed in order to determine the stability of the reversed micelles with respect to temperature and size. Phase behaviour studies showed that the micelles were stable under the conditions employed. The stability diagrams were also broadened with respect to temperature and size by the addition of lysozyme to the micellar water pools.
- Results regarding the forward transfer of native lysozyme showed that pH, contact time, ionic strength and surfactant concentration appeared to be the critical parameters for the solubilisation process. Electrostatic interactions appear to be the dominant driving force for lysozyme solubilisation into these reversed micelles. Decreasing the pH below the pI of lysozyme increased the partitioning of the protein due to an increase in the favourable electrostatic interactions, whereas pH values above the pI drastically reduced the transfer.

At low pH values, the protein was found to denature and unfold and bind to the surfactant molecules irreversibly.

- Lysozyme was seen to loose its tertiary structure as measured by near-uv CD once transferred into the micelles. This denaturation was believed to occur due to the binding of the AOT headgroups to the surface of the protein. This denaturation that took place was found to be completely reversible, since returning the protein back into the aqueous environment returned full activity and three-dimensional structure (Chapter 6).
- The transfer of non-reduced lysozyme into micelles occurs in a similar fashion to the native protein. The maximum protein transfer occurred at a concentration of 1 M GuHCl after which there was a sharp decline in transfer at increased GuHCl concentrations. This was due to a reduction in the size of the micelles which size excluded the proteins from the interior of the micelles. The disulphide bonds contribute to the stability of the non-reduced form of the protein. Structural studies using fluorescence and CD indicated that no major change occurred in the three-dimensional structure of lysozyme upto 3 M GuHCl. Unfolding of the molecule took place between 3-6 M GuHCl and was complete at 6 M GuHCl.
- The transfer of the reduced form of lysozyme into micelles behaved differently than the native and non-reduced forms of the protein. The breaking of the disulphide bonds allow the protein to adapt a more randomly coiled structure. Transfer was found to occur at acidic pH values and this cannot be explained by the electrostatic interactions between the protein and micellar interface. However, these acidic pH values gave the protein alot of negative charge which provided additional binding sites for the positively charged guanidinium ions. These were attracted into the micelles and lysozyme transfer was increased upto a concentration of 3 M GuHCl.
- Investigations were made in an attempt to transfer lysozyme into micelles by changing the denaturant from GuHCl to urea. Under the conditions studied, urea was found to be unsuitable using the phase transfer method and protein concentrations could not be determined due to the viscous natutre of the gels formed.

CHAPTER 5
METHODS TO IMPROVE THE TRANSFER EFFICIENCY OF DENATURED LYSOZYME INTO REVERSED MICELLES

This chapter presents results on two novel methods that have been developed in order to attempt the transfer of denatured lysozyme at higher denaturant concentrations than has been achieved previously in Chapter 4. The first method uses mixed surfactant micelles consisting of AOT and Tween 85 or Tween 20. The second method utilises a mixed denaturant system consisting of mixtures of GuHCl and urea in different ratios.

5.1 The use of mixed micellar systems to attempt to enable the transfer of denatured lysozyme

The optimum GuHCl concentration for reduced lysozyme transfer into reversed micelles is 3 M. However, this optimum denaturant concentration is not high enough to completely unfold lysozyme in an aqueous solution. Above 3 M GuHCl very little or no protein is partitioned into the reversed micelles. This is due to the attractive interactions between the negatively charged surfactant headgroups and the positively charged denaturant which cause the micelle to shrink in size and makes the micelle too small to accommodate the protein. Therefore, it is necessary to develop an alternative method to enable unfolded lysozyme to be transferred into the micelle.

In many practical applications mixtures of surfactants are used which often provide a superior performance to that obtained when using an individual surfactant (Clint, 1994). Household detergents consist of mixtures of anionic and nonionic surfactants to formulate a product that has superior properties to one containing only the anionic or nonionic surfactant.

A number of surfactants were examined in this section including combinations of AOT with the nonionic surfactants Tween 20, Tween 85 and Span 85 systems.

Initially, it requires to be established whether reversed micelles can form by incorporating various amounts of these nonionic surfactants. On the addition of a nonionic surfactant to AOT/isooctane a clear one phase system was determined to be a thermodynamically stable reversed micellar solution. Once the formation of reversed micelles is achieved then the following questions need to be answered. Can the protein be transferred into these new mixed micelle systems under the same conditions as for the AOT micelles? How much noionic surfactant can be put into these mixed micelles? What are the differences in the forward and backward transfer processes?

5.1.2 AOT + Span 85

The AOT/Span 85 system was analysed and proved to be less efficient than the corresponding AOT/Tween systems for the transfer of lysozyme. It was observed that at a concentration of 1% Span 85 in an AOT/Span 85 system, 90% of lysozyme can be transferred into 50 mM AOT/isooctane systems but increasing the concentration of span 85 to 2% only results in a 40% transfer (results not shown). Therefore, as the Span 85 content was increased in the mixed micelle then the transfer of protein decreased. Also increasing the Span 85 concentration resulted in the solution becomming more yellow in colour and made it difficult to measure the protein content in the aqueous phase.Thus, no further experiments were performed using the Span 85 surfactant.

5.1.3 AOT + Tween 85 and AOT + Tween 20 systems

Further experiments were based on a novel idea using mixed surfactant systems consisting of AOT and nonionic surfactants in isooctane to form mixed micelles. The idea was developed as addition of nonionic surfactants to AOT micelles may reduce the number of negatively charged AOT molecules at the micellar interface. Thus, the charge problem associated with the guanidinium ion would be reduced. This could then allow the transfer of protein at higher GuHCl concentrations into the micelles. Also, incorporating these uncharged molecules might lead to an

increase in micellar size. The two nonionic surfactants used were Tween 85 and Tween 20.

The forward transfer of native lysozyme into the AOT:Tween 85 system shows an excellent transfer of 90% protein over the range of mixed micelles used (1-10%) (Figure 5.1). Figure 5.1 shows the forward transfer for the AOT:Tween 20 system as the nonionic component was increased in the mixed micelle system. The results indictae that > 80% of lysozyme was transferred using the AOT:Tween 20 system. It is clear from these results that lysozyme can be placed into these mixed micelles as for the AOT system alone. Since lysozyme can be transferred efficiently using the mixed micelles above, the next step is to determine whether the transfer of denatured protein is able to occur in these novel sytems.

The addition of Tween 85 to AOT in isooctane resulted in the formation of reversed micelles as an optically clear and thermodynamically stable solution was produced. Figure 5.2 shows the transfer of lysozyme in GuHCl into mixed micelle systems consisting of AOT and Tween 85 (24:1) and AOT on its own. The system proved to be less efficient than the AOT system alone. The optimum transfer of protein (90%) for 50 mM AOT + Tween 85 is obtained only between 0.2-0.5 M GuHCl above this concentration of GuHCl there is a sharp decline in protein transfer to 20% at a concentration of 1 M GuHCl. Increasing the mixed surfactant system to 400 mM improves the transfer slightly with 90% transfer occurring at a GuHCl concentration of 1.2 M but drops off to zero at 1.5 M GuHCl. Further increases in surfactant concentration upto 1 M showed no significant improvement in the transfer process (results not shown). The addition of the nonionic surfactant, Tween 85, to the AOT micelles did not improve the transfer efficiency of lysozyme in GuHCl in fact it decreased compared to the AOT system alone. Thus, there must be a change in micellar structure as a result of incorporating these nonionic moieties.

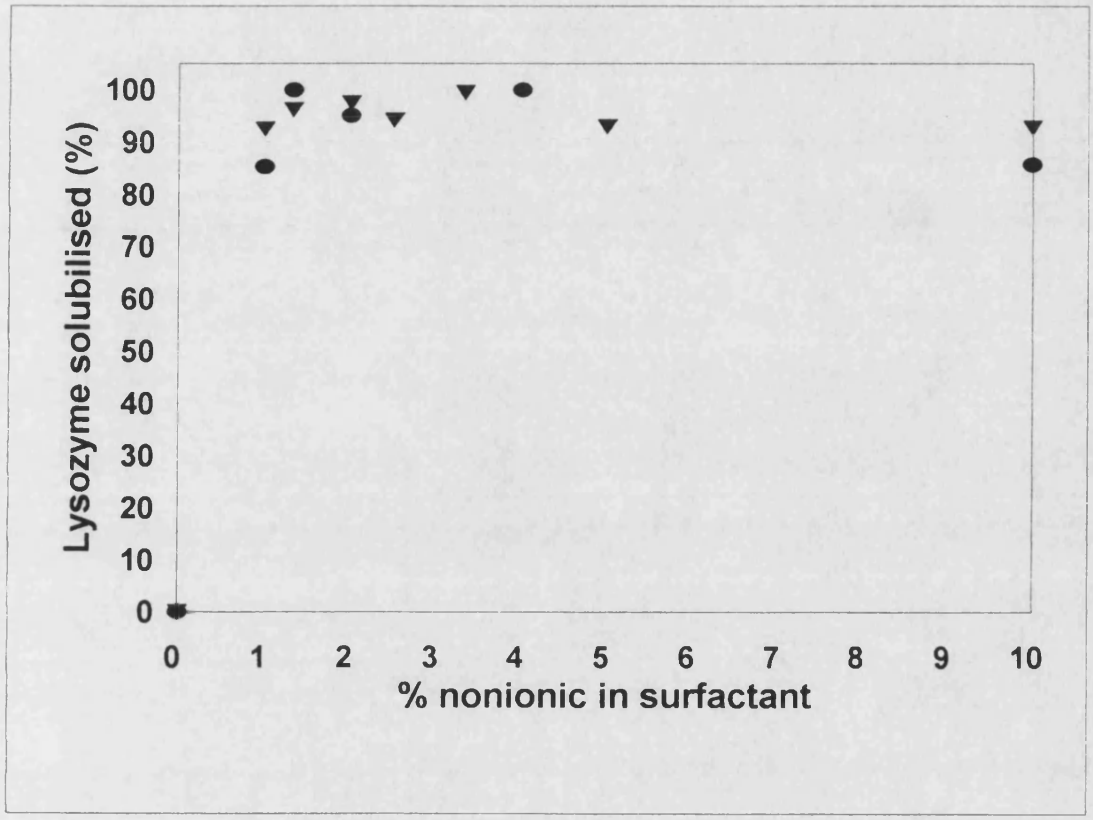

Figure 5.1. The transfer of native lysozyme into mixed micellar systems consisting of AOT and Tween 20 or AOT and Tween 85

The forward extraction of lysozyme involved mixing equal volumes (5 ml) of 50 mM AOT:Tween 20 (●) or AOT:Tween 85 (▼) with an aqueous phase (1 mg/ml lysozyme containing 0.1 M KCl in 25 mM phosphate buffer, pH 7.0) for 10 min. The phases were separated by centrifugation at 2000 rpm for 10 min and the protein concentration in the organic phase was determined.

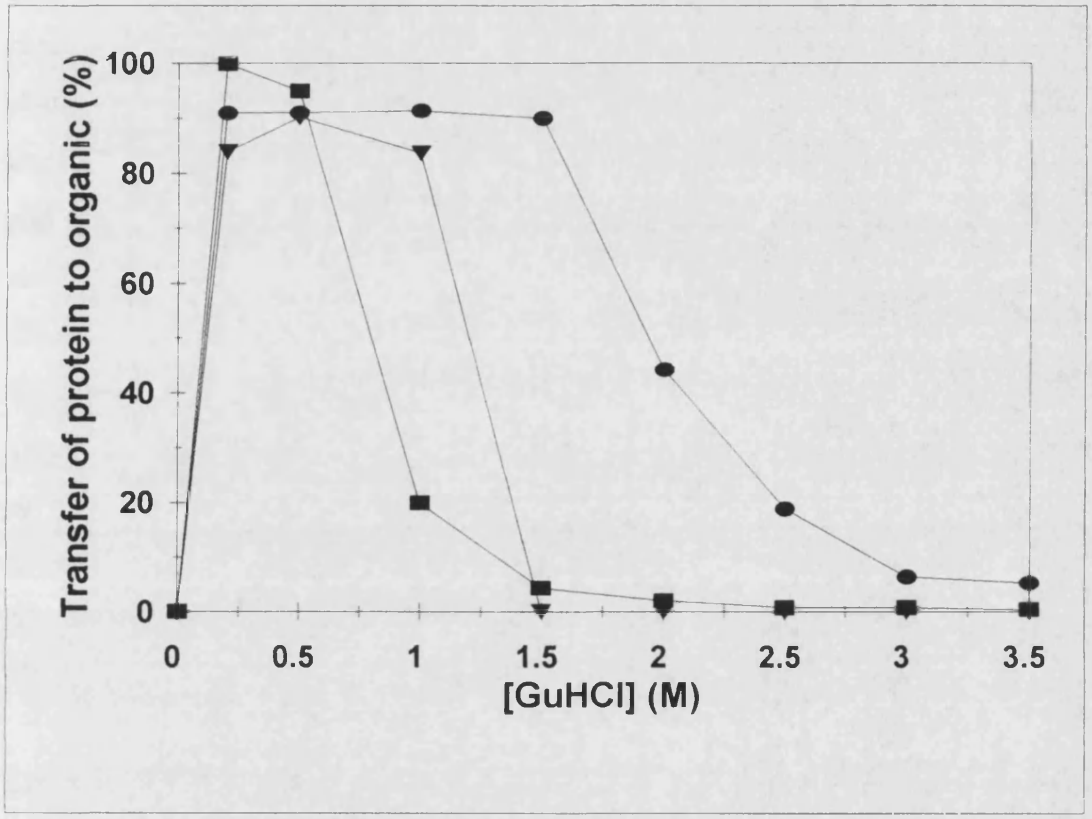

Figure 5.2. The forward transfer of lysozyme into the mixed micellar system consisting of AOT + Tween 85

The forward extraction of lysozyme involved mixing equal volumes (5 ml) of an organic phase consisting of 50 mM AOT + Tween 85 (24:1)/isooctane (■), 400 mM AOT + Tween 85 (24:1)/isooctane (▼) and 400 mM AOT/isooctane (●) with an aqueous phase (1 mg/ml lysozyme in phosphate buffer, pH 7.5) for 15 min. The phases were then centrifuged at 2000 rpm for 10 min to give two clear phases. Protein concentration was determined in the organic phase.

The results presented in Figure 5.2 show that these mixed micelle systems which consist mainly of AOT and with a small amount of Tween 85 (5%) allow poor transfer of lysozyme in GuHCl compared to AOT alone. This decrease in transfer may be attributed to the decreased number of negative charges at the micellar interface. The insertion of Tween 85 to AOT micelles reduces the negative charges present at the micellar interface. This means there are fewer negative charges for guanidine to bind to, which leads to a decrease in the amount of tranferred protein. Yamada *et al.* (1994) studied the extraction of α-chymotrypsin and examined the activity in AOT reversed micelles modified with the nonionic surfactant Tween 85. They found that the extraction of α-chymotrypsin was decreased by the addition of Tween 85 due to the dilution of the negative charge density of the micelles. The activity of α-chymotrypsin was improved by the addition of Tween 85 as it decreased the electrostatic and hydrophobic interactions with the AOT molecule. The mixed micellar system was composed of AOT + Tween 85 (1:1) whereas in the experiments above a 24:1 ratio was used. Tween 85 forms mixed micelles with AOT behaving as a cosurfactant and the shape of the micelles are more elongated than spherical (Yamada *et al.*, 1994).

Dekker (1987) has shown that by addition of a nonionic surfactant (Rewopal HV5) to a reversed micelle phase of TOMAC in octanol + isooctane, the extraction of α-amylase could be improved. The complete extraction of α-amylase from the aqueous phase was made possible by the addition of a small amount of the nonionic surfactant (0.088 % w/v Rewopal HV5). This improvement in transfer of the enzyme may have been caused by changes in the structure of reversed micelles and in their adaptability in size and surface charge density by the addition of the nonionic surfactant.

Hagen (1989) found that the addition of a small amount of the nonionic surfactant BRIJ 30 to an AOT/isooctane system could increase the micellar size by increasing the water content of the micelles. It was thought that the BRIJ 30 may have been acting as a cosurfactant in this system. However, even though the size of micelles increased there was a detrimental effect on the partitioning behaviour.

It was presumed that the BRIJ 30 was forming a bridge with the AOT molecules preventing the transfer of the protein.

It has already been shown that mixed micelles can be formed with AOT and Tween 85 but a reduction in the number of negative charges at the micellar interface also occurs. This leads to a decrease in the amount of lysozyme in GuHCl which can be transferred in comparison with AOT alone. Therefore, there must be a change in the size and adaptibility of these micelles which will be discussed in Section 5.1.4.

The addition of Tween 20 to AOT/isooctane formed optically clear and thermodynamically stable microemulsions. In Figure 5.3 the transfer of lysozyme in GuHCl in a mixed micelle system consisting of AOT + Tween 20 is shown along with results from the AOT system. At a concentration of 0.5 M GuHCl 100% of lysozyme (1 mg/ml) is transferred into the AOT:Tween 20 (19:1) system whereas 17% is transferred at 1 M GuHCl. However, the AOT + Tween 20 (29:1) system transfers 70% lysozyme into the organic phase at a concentration of 1 M GuHCl.

The mixed micelle system is less efficient at transferring lysozyme in GuHCl than the AOT system alone as found for the AOT + Tween 85 system. By increasing the nonionic surfactant concentration in the mixed micellar system the transfer of protein decreases. Therefore the Tween 20 molecule must interfere with the transfer of the protein across the interface. The results presented show that mixed micelles consisting of AOT and Tween 20 behave in a similar way to those consisting of AOT and Tween 85.

The same arguments can be put forward here as was done for AOT+Tween 85. The insertion of the nonionic counterparts to AOT micelles reduces the negative charges present at the micellar interface. This means there are fewer negative charges for guanidine to bind to, which leads to a decrease in the amount of transferred protein.

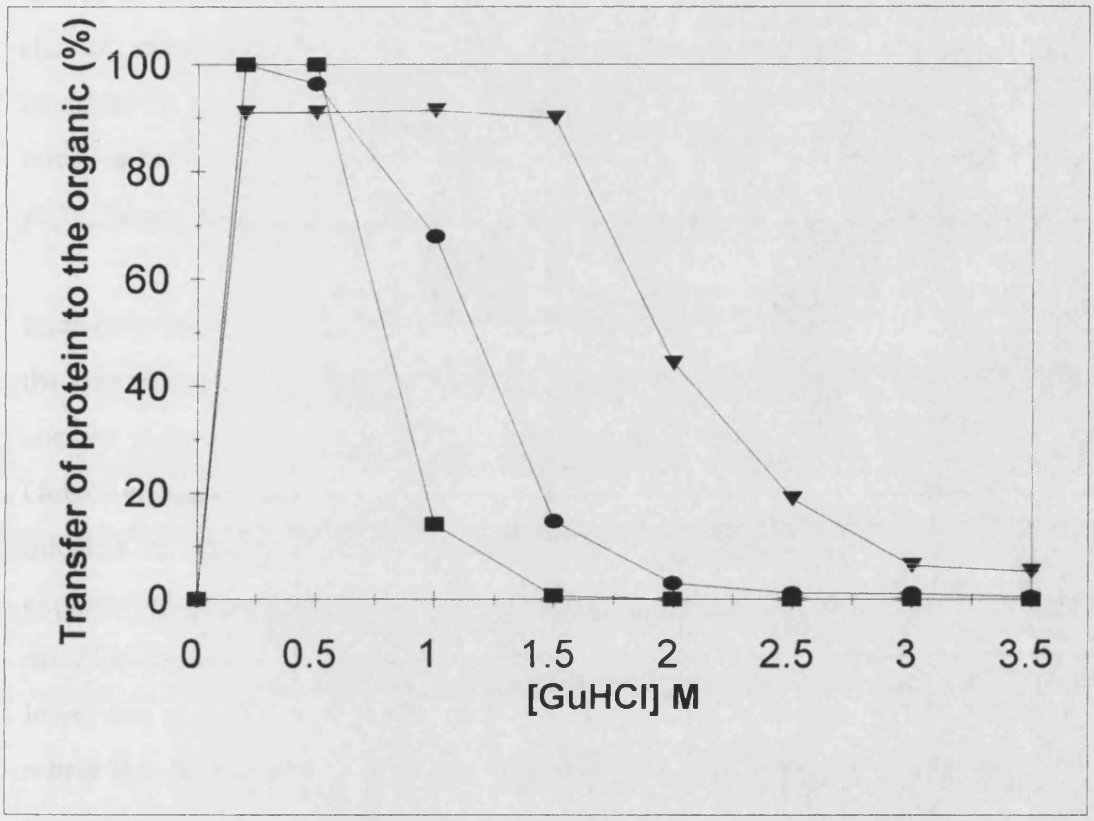

Figure 5.3. The forward transfer of lysozyme into the mixed micellar system consisting of AOT + Tween 20

The forward extraction of lysozyme involved mixing equal volumes (5 ml) of an organic phase consisting of 50 mM AOT + Tween 20 (19:1)/isooctane (■), 50 mM AOT + Tween 20 (29:1/ isooctane (●) and 400 mM AOT /isooctane (▼) with an aqueous phase (1 mg/ml lysozyme in phosphate buffer, pH 7.5) for 15 min. The phases were then centrifuged at 2000 rpm for 10 min to give two clear phases. Protein concentration was determined in the organic phase.

5.1.4 The size of micelles formed with mixed micellar systems

To determine why these mixed micelle systems consisting of AOT and Tween 85 and AOT and Tween 20 transferred less protein than the AOT systems alone, the size of micelles produced with these nonionic surfactants was investigated. The size of micelles formed with various ratios of AOT:Tween 20 systems in isooctane is shown in Figure 5.4. At a concentration of 1 M GuHCl the AOT micelles have a Wo value of about 5 compared to values of 3.2 and 2.5 for the AOT:Tween 20 (29:1 and 14:1) respectively.

Increasing the Tween 20 content in the mixed surfactant system gave a decrease in the size of the micelles. Mixed micelles consisting of AOT and Tween 20 (29:1) contain more water in the micellar core than AOT:Tween 20 (14:1) over the GuHCl concentrations studied. In comparison with the AOT/isooctane system, the micelles of the mixed surfactant systems are much smaller in size and this explains why there is less protein transfer in these new systems. In the mixed micelles used in this work the concentration of the nonionic surfactant is much lower and it is believed that the nonionic components are located at the interface where the AOT is found. The mixed micelles studied had low concentrations of the nonionic surfactants because higher concentrations did not give clear solutions and also poor protein transfer occurred.

5.1.5 Summary

Mixed micelles were formed by the combination of the anionic surfactant AOT with the nonionic surfactants Tween 85 and Tween 20 at low concentrations (1-10 %). A stable microemulsion formed when the solution was optically transparent with no signs of turbidity and was thermodynamically stable. Experiments were performed using these new mixed micelle systems to attempt to improve the transfer of lysozyme in GuHCl into these micelles.

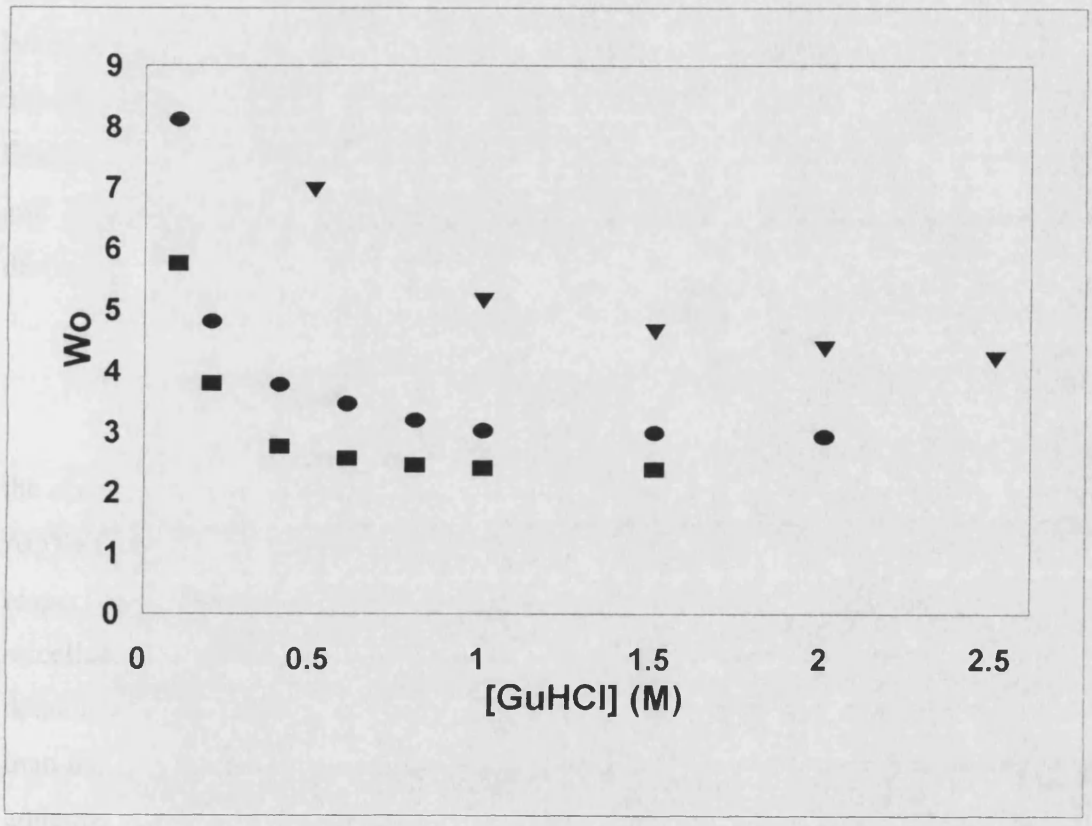

Figure 5.4. The effect of GuHCl concentration on the size of micelles formed in mixed surfactant systems consisting of AOT + Tween 20

Measurement of the water content inside the micelles was studied by mixing equal volumes (5 ml) of an organic phase 50 mM AOT/isooctane (▼), 50 mM AOT + Tween 20 (29:1)/isooctane (●) or 50 mM AOT + Tween 20 (14:1)/isooctane (■) with an aqueous phase (1 mg/ml lysozyme at a particular GuHCl concentration (0.2-2.5 M) in 25 mM phosphate buffer, pH 7.0) for 15 min. The samples were then centrifuged at 2000 rpm for 10 min to give two clear phases. 0.05 ml of the organic phase was injected into the Karl Fischer titrator and the % water per sample was recorded. The average value was taken from 3 determinations.

In both systems the introduction of the nonionic counterpart to the AOT resulted in less lysozyme in GuHCl being transferred into these micelles as compared to the AOT system alone. This was due to the negative charge being reduced at the micellar interface. Thus, there were fewer negative charges present for the guanidine to bind to. The poor transfer is related to the electrostatic interactions between the positive guanidine ion and the negative head groups which cause the micelle to shrink and to expell the protein from the core of the micelle. Karl-Fischer analysis revealed that these mixed micelles had a smaller water content and therefore a smaller size than the AOT system alone. Using the equation derived in Chapter 2 for the radius of the water core of a micelle:

$$\mathbf{r = 0.18 \times Wo} \qquad \textbf{(2.10)}$$

the size of the micelles formed at a concentration of 1 M GuHCl for the AOT, AOT+Tween 20 (29:1) and AOT+Tween 20 (14:1) are 0.92, 0.54 and 0.43 nm respectively. Increasing the nonionic counterpart in these systems caused smaller micelles to be formed which were unable to accommodate the protein as the denaturant concentration was increased. These systems proved to be less efficient than the AOT system alone and thus will not be used to refold proteins inside their aqueous cores. A possible way to enable the transfer of denatured lysozyme into micelles could be to use a completely nonionic micelle. In this case there would be no charge problem associated in the transfer process and the major disadvantage could be overcome.

5.2 The use of mixed denaturants to improve the transfer process

Conditions have been established for the transfer of lysozyme into AOT/isooctane reversed micelles in the presence of the denaturants GuHCl and urea. An upper limit of 2 and 3 M GuHCl for the transfer process into 50 and 400 mM AOT/isooctane reversed micelles exists and above this concentration there is a decline in the amount of protein transferred. In the presence of urea, reversed micelles did not form using the phase transfer method. It was proposed to combine

these denaturants in various ratios in an attempt to reduce the charge problem associated with the positively charged guanidinium ion and the negatively charged AOT headgroups.

The idea of using a GuHCl/urea mixed system to transfer lysozyme into AOT/isooctane micelles has not previously been attempted. Mixtures of denaturants may have synergistic effects on the unfolding of proteins in that the strength of the mixture at a particular concentration may be greater than the strength obtained by the individual denaturant (Lilley and Tester, 1982). This section examines whether micelles can be formed using mixtures of GuHCl and urea and if so the limits of the transfer of lysozyme under the conditions employed.

5.2.1 The effect of various GuHCl:urea ratios on the forward transfer of non-reduced and reduced lysozyme

All the mixtures of GuHCl and urea tested formed reversed micelles on contacting with the organic phase. A reversed micellar phase was observed as an optically clear and thermodynamically stable solution with no signs of turbidity. The transfer of lysozyme in mixed denaturant solutions and the transfer of lysozyme in GuHCl only at several concentrations are shown in Figure 5.5. A mixture of GuHCl:urea (1:1) shows no improvement on protein transfer as compared to the GuHCl system, both having similar curves. Both conditions give a maximum protein transfer of around 90% at 1.5 M denaturant. By reducing the GuHCl content compared to urea in the mixtures (2:3 and 1:4) it was found that the protein could be transferred at higher mixed denaturant concentrations. A GuHCl:urea ratio of 2:3 gives a transfer of about 90% at a total concentration of 3 M and at a ratio of 1:4 high transfer of protein occurs at a concentration upto 5 M. In each case the concentration above where the maximum transfer occurs the amount of protein transferred was found to decline. The mass balance between the organic and aqueous phases gave an error of less than 3%. The results indicate that reducing the GuHCl content relative to the urea content in a mixed denaturant

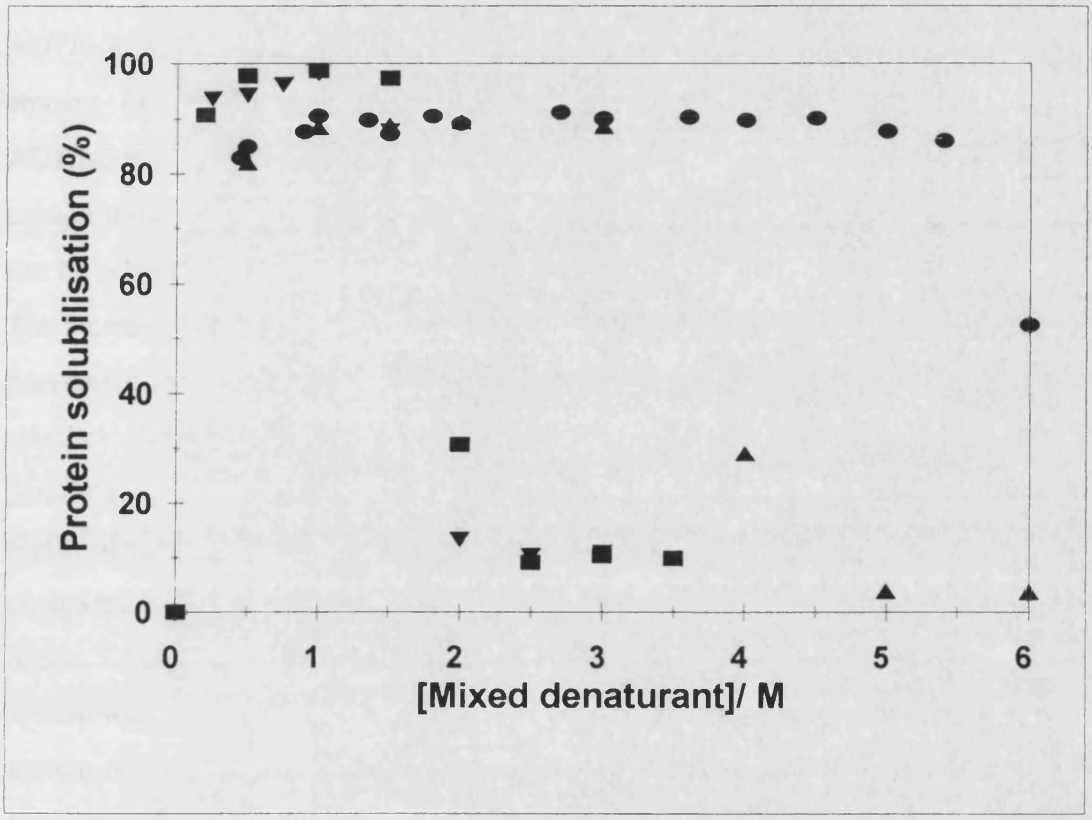

Figure 5.5. The effect of mixed denaturants (GuHCl/urea) on the forward transfer of non-reduced lysozyme into 400 mM AOT/isooctane micelles

The forward extraction of lysozyme involved mixing equal volumes of an organic phase (400 mM AOT/isooctane) with an aqueous phase (1 mg/ml lysozyme in 25 mM phosphate buffer, pH 7) at various mixed denaturant concentrations of GuHCl:urea, 1:1 (▼), 2:3 (▲), 1:4 (●) and GuHCl (■). The mixing time was 15 min then the samples were centrifuged at 2000 rpm for 10 min giving two clear phases. Protein concentration was determined in both phases using A_{280} measurements.

system can allow the transfer of lysozyme into AOT/isooctane micelles at a much higher concentrations than the GuHCl system alone.

Table 5.1 summarises the transfer of non-reduced lysozyme into AOT/isooctane reversed micelles systems using GuHCl and urea mixtures. For 50 mM AOT/isooctane reversed micelle systems with a GuHCl:urea ratio of 0.5:4.5 the amount of protein transferred (90%) is greater than with the 400 mM AOT/isooctane micelle systems (>80%). Even though there is a decline of 10% in protein transfer at the higher surfactant concentration (400 mM), protein transfer can be achieved at higher denaturant concentrations i.e. 7 M as compared to 6 M. The increase in protein transfer at higher denaturant concentrations was explained previously by the fact that increasing surfactant concentration results in larger micelles. When the alkane phase was changed from isooctane to n-octane in the presence of GuHCl:urea 0.5:4.5, the protein was found to be transferred at a higher denaturant concentration (8 M). Thus, using the straight chain alkane compared to the branched chain alkane enables protein transfer to occur at even higher denaturant concentrations. This result highlights another aspect of the system which could be investigated. In the GuHCl:urea (0.2:4.8) mixed denaturant system 60% of the protein partitioned into the micelle at a concentration of 8 M, however, the organic phase was slightly viscous. This was due to large amount of urea present in the mixture. Table 5.2 summarises the transfer of reduced lysozyme into AOT/isooctane reversed micelle systems using GuHCl and urea mixtures. The transfer of lysozyme is observed to partition at higher mixed denaturant concentrations than with the non-reduced form.

It has been found that lysozyme cannot be partitioned into the AOT/isooctane micelles when using urea as the denaturant (Section 4.5). Several experiments were performed using either different mixing times, salt (KCl) concentrations or alkanes ranging from hexane to hexadecane. However, the forward transfer of lysozyme into organic phase resulted in the formation of viscous gels in all the conditions examined. Experiments were also conducted in the absence of the protein to determine whether the protein was involved in the formation of the gels.

Table 5.1. A comparison of the transfer of non-reduced lysozyme into AOT/isooctane reverse micelles using a number of mixed denaturants.

Denaturant System	Maximum Denaturant Concentration	Maximum Protein Transfer	Organic Phase Conditions
Urea with 0.1M KCl	1-8 M	None	50-400 mM AOT/isooctane
GuHCl	1.8 M	~90 %	400 mM AOT/isooctane
GuHCl : Urea 1:1	1.5 M	~86 %	400 mM AOT/isooctane
GuHCl:Urea 2:3	2 M	90 %	50 mM AOT/isooctane
GuHCl : Urea 2:3	3 M	>82 %	400 mM AOT/isooctane
GuHCl:Urea 1:4	4 M	90 %	50 mM AOT/isooctane
GuHCl : Urea 1:4	5.4 M	>82 %	400 mM AOT/isooctane
GuHCl:Urea 0.5:4.5	6 M	90 %	50 mM AOT/isooctane
GuHCl : Urea 0.5:4.5	7 M	>80 %	400 mM AOT/isooctane
GuHCl:Urea 0.5:4.5	8 M	80 %	400 mM AOT/octane
GuHCl:Urea 0.3:4.7	7.5 M	80 %	400 mM AOT/isooctane
GuHCl : Urea 0.2:4.8	8 M	60 %	400 mM AOT/isooctane

Table 5.2. A comparison of the transfer of reduced lysozyme into AOT/isooctane reversed micelles using a number of mixed denaturants.

Denaturant System	Maximum Denaturant Concentration	Maximum Protein Transfer	Organic Phase Conditions
GuHCl : Urea 1:1	1.8 M	85 %	50 mM AOT/isooctane
GuHCl:Urea 1:1	2 M	82 %	400 mM AOT/isooctane
GuHCl : Urea 2:3	2.5 M	85 %	50 mM AOT/isooctane
GuHCl:Urea 2:3	4 M	83 %	400 mM AOT/isooctane
GuHCl : Urea 1:4	5 M	86 %	50 mM AOT/isooctane
GuHCl:Urea 1:4	6 M	82 %	400 mM AOT/isooctane
GuHCl : Urea 0.5:4.5	6.5 M	88 %	50 mM AOT/isooctane
GuHCl:Urea 0.5:4.5	8 M	85 %	400 mM AOT/isooctane
GuHCl:Urea 0.3:4.7	9.5 M	83 %	400 mM AOT/isooctane
GuHCl : Urea 0.2:4.8	10.5 M	70 %	400 mM AOT/isooctane

The same results were achieved in both the absence and presence of therefore the protein was not involved in gel formation.

This new mixed denaturant system is effective for the transfer of non-reduced lysozyme into AOT/isooctane micelles. The concentrations at which transfer can occur in these mixed systems are higher than can be achieved when using either GuHCl or urea individually. Reducing the disulphide bonds of lysozyme and allowing it to have a less rigid structure should increase the denaturant concentration at which the protein could partition into the micelles. This is explained by opening of the molecule making available more binding sites for the denaturant molecules and this possibly facilitates transfer of protein at even higher denaturant concentrations. Table 5.2 summarises the transfer of the reduced form of lysozyme into reduced form of the protein into AOT/isooctane micelles (50-400 mM). The mass balance of the protein in the organic and aqueous phases after the forward transfer gave an error of less than 3%. For a GuHCl:urea (0.5:4.5) mixture the non-reduced protein transfers into a 50 mM AOT/isooctane micelle (90%) at a concentration of 6 M. For the same mixture the reduced form of the protein transfers (88%) at a concentration of 6.5 M.

There are many questions that arise from this work with the mixed denaturants. The first one is to what extent can they unfold lysozyme? This will be answered in Section 5.2.3 and 5.2.4 which looks at the structure of lysozyme in certain mixtures of these denaturants. Also, what are the sizes of these micelles formed using mixtures of GuHCl and urea? These results will be presented in the next section.

5.2.2 The size of micelles formed with mixed denaturants

The size of micelles formed using several mixed denaturant systems is shown in Figure 5.6. At a mixed denaturant concentration of 3 M the Wo value for GuHCl:urea (0.5:4.5) is about 14 compared to a value of 5 for the GuHCl:urea (2:3) system. The micelles formed during the transfer of lysozyme using only

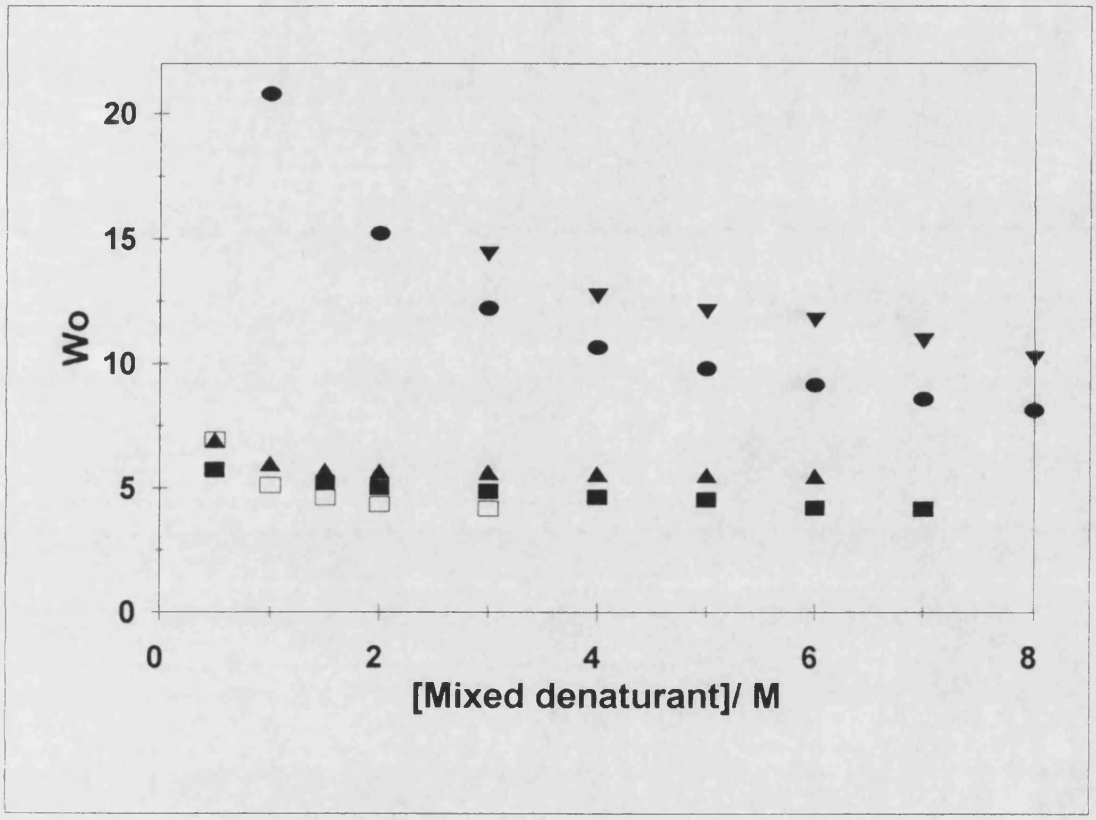

Figure 5.6. The size of micelles formed using the mixed denaturant systems

Measurement of water content inside the micelles was studied by mixing equal volumes of an organic phase (400 mM AOT/isooctane) with an aqueous phase (1 mg/ml lysozyme in 25 mM phosphate buffer, pH 7.0) in a particular denaturant, GuHCl (□), GuHCl:urea (2:3) (■), GuHCl:urea (1:4) (▲), GuHCl:urea (0.5:4.5) in isooctane (●) and GuHCl:urea (0.5:4.5) in n-octane (▼). The samples were mixed for 15 min then they were centrifuged at 2000 rpm for 10 min. 0.05 ml of the organic phase was injected into the Karl-Fischer titrator and the % water was measured. The results are an average value from three determinations.

GuHCl are of the smallest size. In the mixed denaturant systems as the GuHCl content decreases and the urea content increases the micelles become larger in size. The results show that the positively charged guanidinium ion causes the micelles to become smaller in size. Since urea is an uncharged molecule there are no interactions between the negatively charged surfactant head groups. Thus, the lower the GuHCl content in these systems, the larger the micelles formed and hence the greater the transfer of protein.

The size of the micelles formed with the mixed denaturant system consisting of GuHCl:urea (0.5:4.5) using either isooctane or n-octane are compared in Figure 5.6. By changing the oil phase from isooctane to n-octane the micelle size increases from a Wo value of 8 to 10 at a concentration of 6 M. This explains why lysozyme is transferred at a higher denaturant concentration with n-octane than isooctane (Table 5.1). The result is unexpected as there is only a slight change in the structure between the two oils, isooctane is branched and n-octane is straight chained. These results reveal how slight changes in the oil phase can change the size of the reversed micelles formed.

5.2.3 Structural state of lysozyme in various mixtures of GuHCl and urea measured by fluorescence

It has been shown that lysozyme can be partitioned into reversed micelles at certain mixed denaturant concentrations (Table 5.1). However, the strength of the mixed denaturant solutions and the extent to which they unfold lysozyme is unknown. Fluorescence measurements can reveal what effect these mixed denaturant solutions have on the tertiary structure of lysozyme and therefore the extent of unfolding.

The fluorescence emission spectra between 300-400 nm of mixtures of denaturants (GuHCl and urea) at various ratios and concentrations were recorded. The fluorescence at 350 nm of lysozyme in various mixtures of GuHCl and urea are shown in Figure 5.7. The 1:1 ratio of GuHCl:urea appears to unfold lysozyme

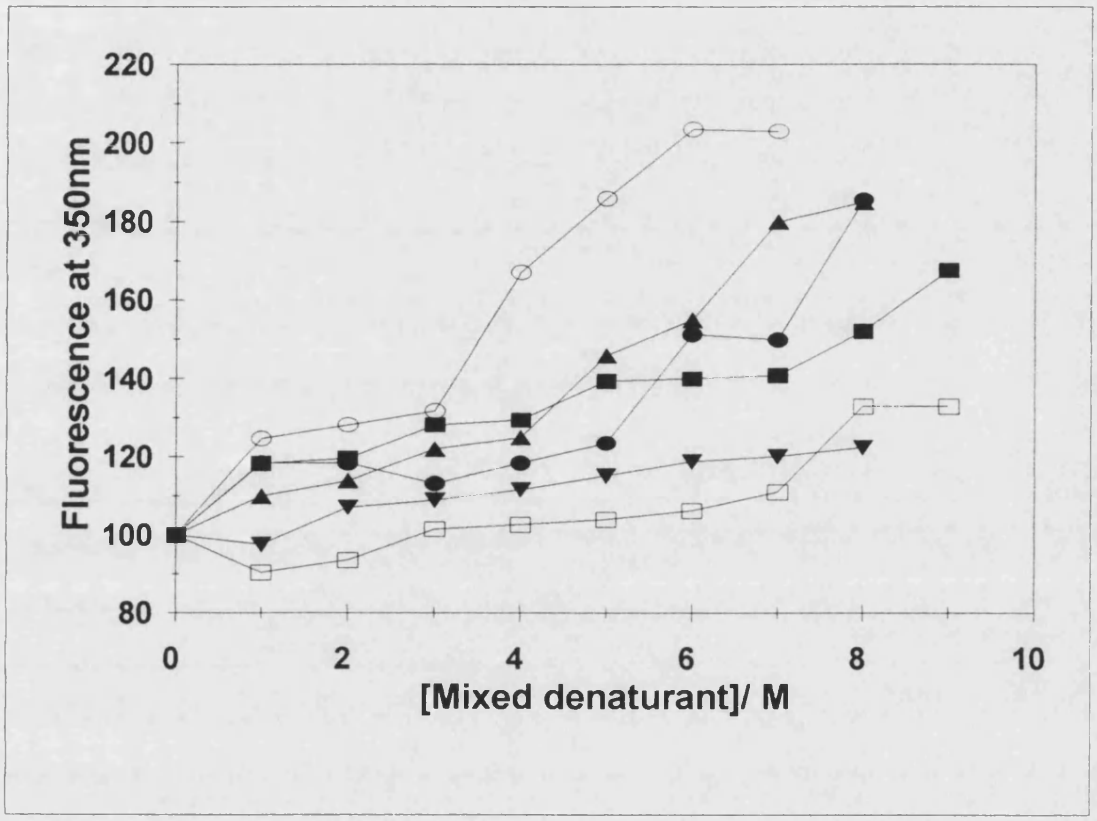

Figure 5.7. Fluorescence at 350 nm of lysozyme in various mixtures of GuHCl and urea

Lysozyme (10 μg/ml) was incubated in various mixtures of GuHCl and urea, GuHCl:urea (1:1) ●, (1:4) ■, (0.5:4.5) ▲ and (0.2:4.8) ▼for 20 min. Samples (1 ml) were excited at 290 nm. The data for GuHCl (○) and urea (□) have been included for comparison. The emission spectra were recorded between 300-400 nm at several denaturant concentrations. The relative fluorescence at 350 nm are plotted using arbitary units.

to the greatest extent, especially at a concentration of 8 M having a relative fluorescence of about 180. This value is fairly close to that obtained for lysozyme in a 6 M GuHCl solution (200). The results indictate that for the ratios chosen (0.2:4.8, 0.5:4.5, 1:4 and 1:1) the unfolding power is greater than can be achieved by using urea alone but they effect unfolding to a lesser extent than GuHCl. At this 1:1 ratio the denaturants are probably behaving synergistically and the extent of the unfolding is superior to that of the individual denaturants. However, the 1:1 ratio of the mixed denaturant is unable to transfer lysozyme into AOT/isooctane micelles as it has an upper limit for the transfer at about 1.8 M (Section 5.2.1). The other three mixed denaturant systems affect the tertiary structure of lysozyme to a lesser extent than the 1:1 ratio with the 0.2:4.8 system unfolding lysozyme in a similar fashion to urea since it is predominantly urea.

Fluorescence measurements above examined the unfolding of lysozyme in the non-reduced form. In the reduced form, when the disulphide bonds are broken, it is likely the protein would unfold to a greater extent as it is not restricted by the disulphide bonds. These results indicate that mixed denaturant solutions can unfold lysozyme. The fluorescence values for the mixed denaturant systems are between the values achieved for urea and GuHCl individually and confirm that GuHCl unfolds lysozyme to a greater extent than urea.

5.2.4 Structural state of non-reduced and reduced lysozyme in various mixtures of GuHCl and urea using CD in the near-uv region

The fluorescence data in the previous section shows that the mixed denaturant systems examined unfold lysozyme to a greater extent than can be achieved by urea alone. The CD in the near-uv for several of the mixed denaturant systems was analysed.

The CD spectra of non-reduced and reduced lysozyme after the forward transfer with GuHCl:urea (0.5:4.5 and 1:4) into 50 mM AOT/isooctane reversed micelles

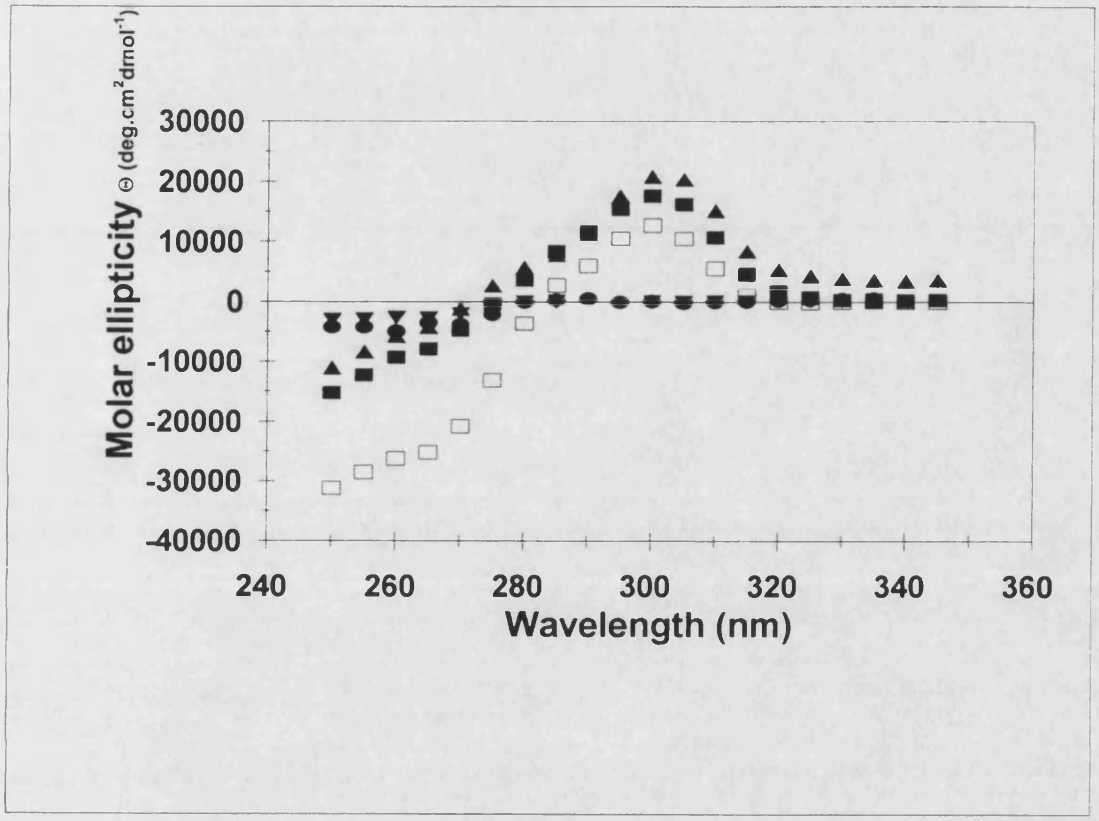

Figure 5.8. Circular dichroism spectra of non-reduced and reduced lysozyme in systems consisting of GuHCl:urea in 50 mM AOT/isooctane micelles

Near-uv Circular dichroism spectra were recorded for non-reduced lysozyme in a GuHCl:urea (0.5:4.5) 6 M (■) and (1:4) 5 M (▲) after the forward transfer into 50 mM AOT/isooctane micelles which yielded 90% of the protein in the reversed micellar phase. Spectra were also recorded for reduced lysozyme in GuHCl:urea (0.5:4.5 M) ▼ and (1:4 M) ● and compared to native lysozyme (□). The samples were left for 30 minutes before spectra were recorded between 250-340 nm. The spectra were recorded at a scanning rate of 2 nm/min and are an average of 3 determinations after subtraction of the buffer blanks.

are shown in Figure 5.8. The data for native lysozyme is included for comparison. The characteristic peak between 280-300 nm is observed for both the non-reduced samples which have molar ellipticity values of between 15000-20000 whereas native lysozyme has a molar ellipticity value of 10000. Exposing lysozyme to the mixed denaturant systems results in a slight change in the tertiary structure of the protein. There was no loss in the tertiary structure of lysozyme in the mixed denaturant system as the peak between 280-300 nm did not disappear. CD measurements were also performed for reduced lysozyme in the mixed denaturant systems as shown in Figure 5.8. In both the reduced samples lysozyme appeared to be unfolded due to the complete loss in the tertiary structure as the molar ellipticity values remained zero over the entire wavelength (250-340 nm). This reveals that by reducing the disulphide bonds of the protein, the denaturant is able to bind more easily to the protein and thus, unfold it at a lower denaturant concentration.

Figure 5.8 also shows that the plateau region at 260 nm for the mixed dentaurant systems is very different to that of the native one. For the native lysozyme a molar ellipticity value of -30000 occurs at 260 nm whereas for the mixed denaturant systems molar ellipticity values of -10000 and above are recorded. This indicates that some changes are occurring in the three-dimensional structure of lysozyme in the presence of the mixed denaturant systems.

Reduced lysozyme in several of the mixed denaturant systems at high concentrations was found to unfold lysozyme at lower concentrations than urea. The advantage of the mixed denaturant systems is that protein can be transferred into AOT/isooctane reversed micelles at higher denaturant concentrations than can be achieved by using GuHCl alone. The transfer process has been improved to allow transfer of denatured lysozyme and the next stage is to attempt to reoxidise and refold the protein inside the micelle before back extracting the protein into the aqueous environment.

5.2.5 Summary

The results presented in this section describe a novel method for the partitioning of lysozyme (non-reduced and reduced) into AOT/isooctane micelles using mixtures of GuHCl and urea in various ratios. The mixed denaturant systems at certain ratios have been able to partition lysozyme at higher denaturant concentrations than can be achieved when GuHCl is used as the denaturant. This is due to urea being an uncharged molecule and since the mixed denaturant systems can contain a greater quantity of urea than GuHCl then the charge problem associated with GuHCl is avoided. However, at very high concentrations of mixtures of GuHCl and urea, the electrostatic interactions between GuHCl and the AOT head groups do exist and thus, causes smaller micelles to be formed which size excludes the protein from the micelle.

Transfer of reduced lysozyme is able to occur at higher mixed denaturant concentrations the non-reduced lysozyme as the more open structure allows more binding sites for both the GuHCl and urea. The presence of a greater amount of bound denaturant to the reduced protein facillitates transfer into the micelle more easily than for the non-reduced protein.

The strength of the mixed denaturant systems by monitoring changes in using fluorescence and CD was investigated in the structure of the protein in different denaturant concentrations. Fluorescence studies indicated that the mixed denaturant systems were able to unfold lysozyme more effectively than urea but they were not effective as GuHCl. CD data showed non-reduced lysozyme had a slightly different structure than native lysozyme. However, reduced lysozyme in the mixed denaturant systems studied was found to completely lose its tertiary structure.

CHAPTER 6
REFOLDING LYSOZYME

This chapter presents results on refolding lysozyme in an aqueous environment and results on refolding the protein inside micelles. The chapter also describes the backward transfer process required to return the protein from the reversed micelles into an aqueous environment.

6.1 Refolding lysozyme in an aqueous environment

This section investigates the parameters which influence the refolding of non-reduced and reduced lysozyme in an aqueous environment. The parameters studied were temperature and protein concentration. These experiments form the basis against which reversed micelle refolding may be compared.

6.1.1 Inactivation of lysozyme

In order to establish the denaturant concentration at which enzyme activity is lost, inactivation studies were performed at various GuHCl concentrations (Figure 6.1). At 0.1 M GuHCl the protein has about 60% of its activity, increasing the denaturant concentration to 0.2 M reduces the activity to about 10% and at 0.3 M less than 5% activity is retained. This clearly illustrates that the amount of denaturant used to inactivate lysozyme is much lower than the concentration required to significantly alter protein structure or to solubilise inclusion bodies. The fluorescence data shown earlier (Section 4.3.1.2) indicated that there were only small changes in the structure of the protein at concentrations below 3 M GuHCl but at higher concentrations (3-6 M) there were large structural changes resulting in unfolding of the protein molecule. The low concentrations of GuHCl used in these inactivation studies led to complete loss in activity of the protein and since no major conformational change was observed any change must take place at the active site of lysozyme. Lysozyme is a robust molecule as high denaturant

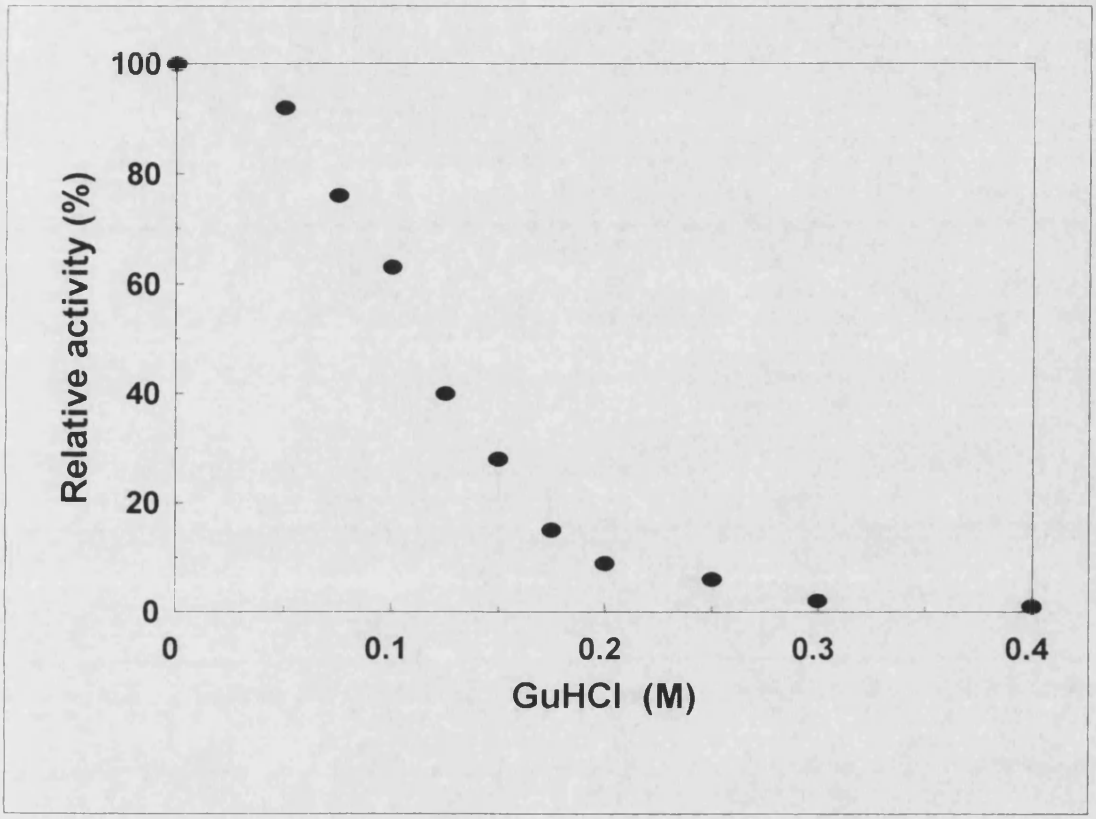

Figure 6.1. Inactivation of lysozyme in GuHCl

Lysozyme (0.5 mg/ml) was incubated in 0.06 M potassium phosphate, pH 6.2 in the presence of increasing concentrations of GuHCl (0.05-0.4 M) at 20°C for 15 min. Enzyme activity was assayed in the same concentration of GuHCl and expressed relative to a control sample from which GuHCl was omitted.

concentrations are required to unfold the protein (Steiner, 1964). This indictaes that the tertiary structure of the protein is held together by strong forces and high concentrations of denaturant are requred to break these forces or bonds. However, since low concentrations of GuHCl inactivate lysozyme, the bonds at the active site of the protein must be very weak and easily broken. The active form of the protein is held together by a delicate balance of non-covalent forces. By exposing it to the denaturing conditions shown, these non-covalent forces are first weakened and then broken apart. This transition to a different conformation disassembles the active site of lysozyme and causes inactivation.

Zhou *et al.* (1993) have reported the denaturation of creatine kinase by GuHCl or urea and have shown that inactivation of the enzyme occurs before any noticeable conformational change of the molecule as a whole. They used a fluorescent probe formed near the active site of the protein and the inactivation and exposure of this probe took place simultaneously well before the unfolding of the molecule as a whole. This is probably due to the fact that the conformation of the enzymes active sites are held together by relatively weak forces and are easily perturbed by chaotropic reagents. Similar results have been obtained by Wang *et al.* (1995) with creatine kinase in SDS solutions.

6.1.2 Refolding non-reduced lysozyme

Lysozyme in the non-reduced state with its disulphide bonds remaining intact has been examined by CD and fluorescence in Sections 4.3.1.2 and 4.3.1.3. The results showed that a concentration of approximately 4 M GuHCl was required to disrupt the tertiary structure. However, concentrations of urea, even at 10 M, did not appear to alter the tertiary structure of lysozyme to the same extent as GuHCl. These results show that the presence of the four disulphide bonds in lysozyme contribute to its stability. Breakage of the disulphide bonds results in the loss of tertiary structure at lower denaturant (GuHCl and urea) concentrations (Section 4.4.3). When the disulphide bonds remain intact there are constraints on the structure of the protein thus some structure is maintained in the unfolded state.

Figure 6.2 shows the refolding of denatured, non-reduced lysozyme as a function of GuHCl and urea concentration. For both chaotropic reagents over the entire concentration range, full lysozyme activity is regained. Diluting the denatured protein into refolding buffer (100-fold) reduces the denaturant concentration to less than 0.1 M and this allows the protein to refold into its native conformation. The presence of disulphide bonds generally stabilize protein conformation and when they are maintained in the unfolded protein then the unfolding is usually reversible and refolding generally occurs rapidly Creighton (1990). These bonds severely limit the possible conformations accessible to the randomly coiled chain and favour refolding by limiting the formation of intermediates other than those leading more directly to the native conformation. This then makes the process rapid as shown in Figure 6.2 since activity is immediately regained.

For both urea and GuHCl denatured lysozyme full activity was regained and thus its three-dimensional structure formed by simply diluting the denaturant concentration. However, the dilution required is large and the amount of denaturant remaining should be less than 0.05 M in the case of GuHCl, since above this concentration the activity declines rapidly (Figure 6.2).

6.1.3 Refolding reduced lysozyme

The refolding of lysozyme from the non-reduced state, denatured in GuHCl or urea, was found to recover 100% activity by simply diluting the denaturant. For disulphide bond containing proteins strong denaturants are used to completely solubilise the protein. Any misfolded disulphide bonds must be broken. To refold the protein, a simple dilution does not return the protein to its native form. The reoxidation of the disulphide bonds is also required to form the three-dimensional structure of the protein.

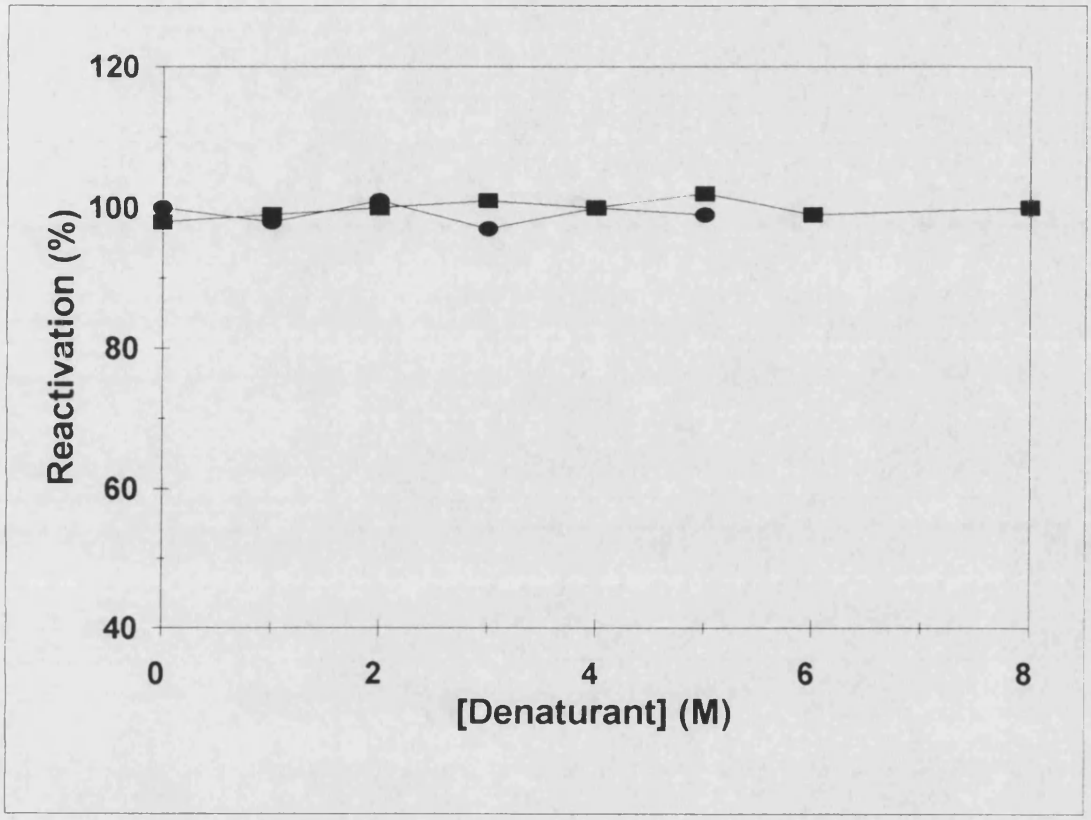

Figure 6.2. Reactivation of non-reduced lysozyme from GuHCl and urea in an aqueous environment

Lysozyme (1 mg/ml) was incubated in 0.06 M potassium phosphate, pH 6.2 in the presence of 1-6 M GuHCl (●) and 1-8 M urea (■) at 20 °C for 15 min. Reactivation of the enzyme solution was initiated by adding a sample of the denatured protein to the renaturation buffer (0.1 M Tris-HCl, pH 8.2). Enzyme activity was expressed relative to native lysozyme at the same concentration.

Figure 6.3 shows the refolding of lysozyme (0.015 mg/ml) in an aqueous solution. Lysozyme can be refolded to gain about 65% of its native activity. The recovery of about 50% activity can be achieved within the first 10 min. After 60 min no further recovery of activity was obtained.

The conditions used for disulphide bond formation were optimised previously by Saxena and Wetlaufer (1970). The maximum rate of refolding was found when using the ratio of oxidised to reduced glutathione was (GSSG/GSH) 1:10. Saxena and Wetlaufer (1970), found that the maximum rate obtained required less than 5 minutes for 50% regeneration and at 30 min the yield had increased to 60-85 % of the native lysozyme activity. The correct number of disulphide bonds were reformed as confirmed by Ellmans assay. Goldberg *et al.* (1991) have shown similar results to those obtained in Figure 6.3 for the refolding of denatured and reduced lysozyme.

It is believed that a competition exists between refolding and aggregation and for the folding reaction to dominate the protein concentrations must be kept as low as possible. It is due to this aggregation side reaction that the yield of refolded protein dramatically decreases. Protein aggregation is the result of intermolecular interactions occurring between exposed hydrophobic residues between individual protein molecules.

It is due to the presence of the aggregation side reaction which occurs that there is a potential for reversed micelles to refold lysozyme. Firstly, reversed micelles allow the protein molecules to be isolated from one another thus preventing aggregation and secondly, the protein concentration at which refolding takes place can be increased to greater concentrations (1 mg/ml) than in the aqueous environment (μg/ml).

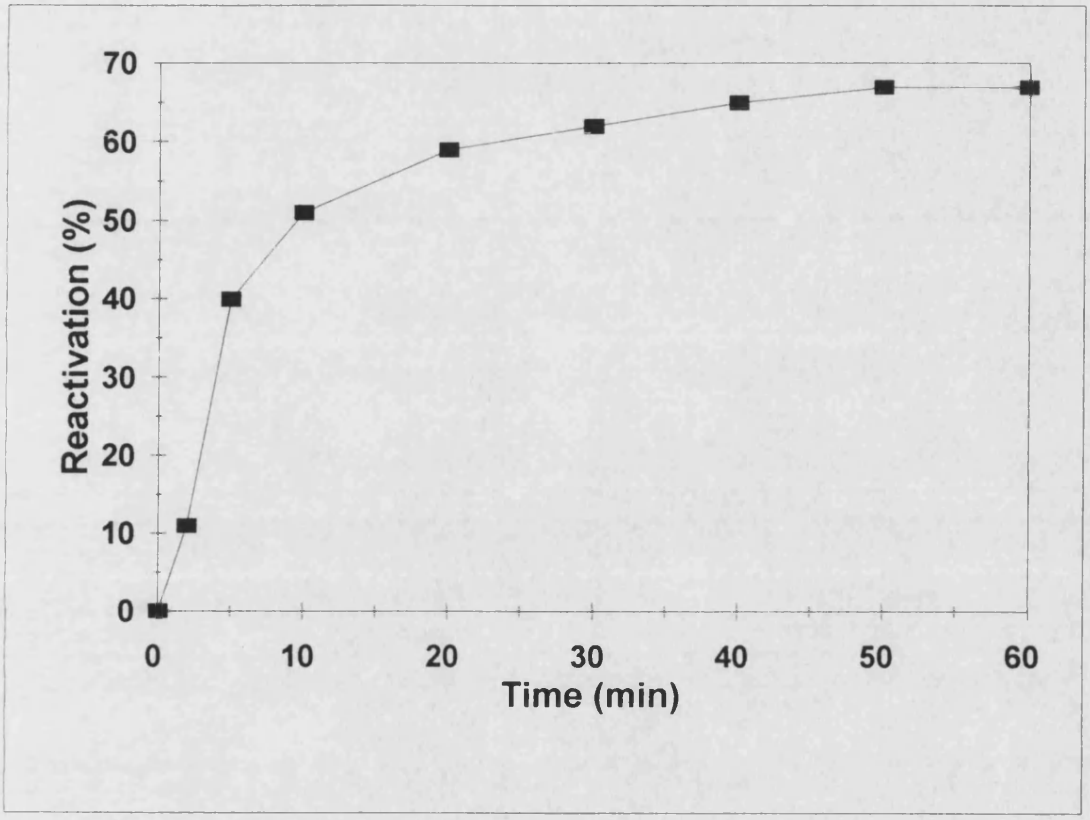

Figure 6.3. Refolding denatured and reduced lysozyme

Lysozyme was reduced and denatured in 6 M GuHCl. Renaturation-reoxidation of the enzyme solution was initiated by adding denatured and reduced lysozyme to a final concentration of 0.015 mg/ml to renaturation buffer (0.1 M Tris-HCl, pH 8.2, 1 mM EDTA, 3 mM reduced glutathione and 0.3 mM oxidised glutathione) at 40°C. Enzyme activity was assayed at several time points and expressed relative to native lysozyme at the same concentration.

6.1.3.1 The effect of temperature on refolding

An important parameter that can be altered to maximise refolding is temperature. Figure 6.4 shows the refolding of denatured and reduced lysozyme as a function of temperature. At 10°C there is a reduction in the activity recovered as only about 30% is achieved compared to 65% at 40°C. Increasing the temperature to 60°C did not improve the recovery of activity and a similar value to that obtained at 40°C was observed. The results presented in Figure 6.4 show that the rate of reactivation at the lower temperature of 10°C is a lot slower than that at 40°C and 60°C as can be seen by the slope of the curves. The rate of renaturation of proteins from denatured and reduced material involves a competition between renaturation and aggregation.

Fischer *et al.* (1993) have studied the renaturation of lysozyme as a function of temperature. They found that by increasing the temperature to 50°C the renaturation yield and renaturation rate constant increased while the formation of aggregated protein decreased. They postulated that increasing the temperature decreased the accumulation of hydrophobic intermediates and the formation of insoluble aggregates thus the recovery of the active protein increased. Increases in temperature resulted in an increase in activity which meant a decrease in aggregation. The aggregation is caused by hydrophobic interactions between exposed hydrophobic residues on the surfaces of the protein molecules. By increasing temperaure it is postulated that the burial of these hydrophobic residues is accelarated resulting in higher activity of the protein.

The conditions employed by Fischer *et al.* (1993) were exactly the same as for the results presented here but the discrepancy cannot be accounted for. However, the results in this section agree with the data obtained by Saxena and Wetlaufer, 1970. The results indicate that the optimum temperature for maximum reactivation of reduced lysozyme is 40°C where approximately 65% activity is regained.

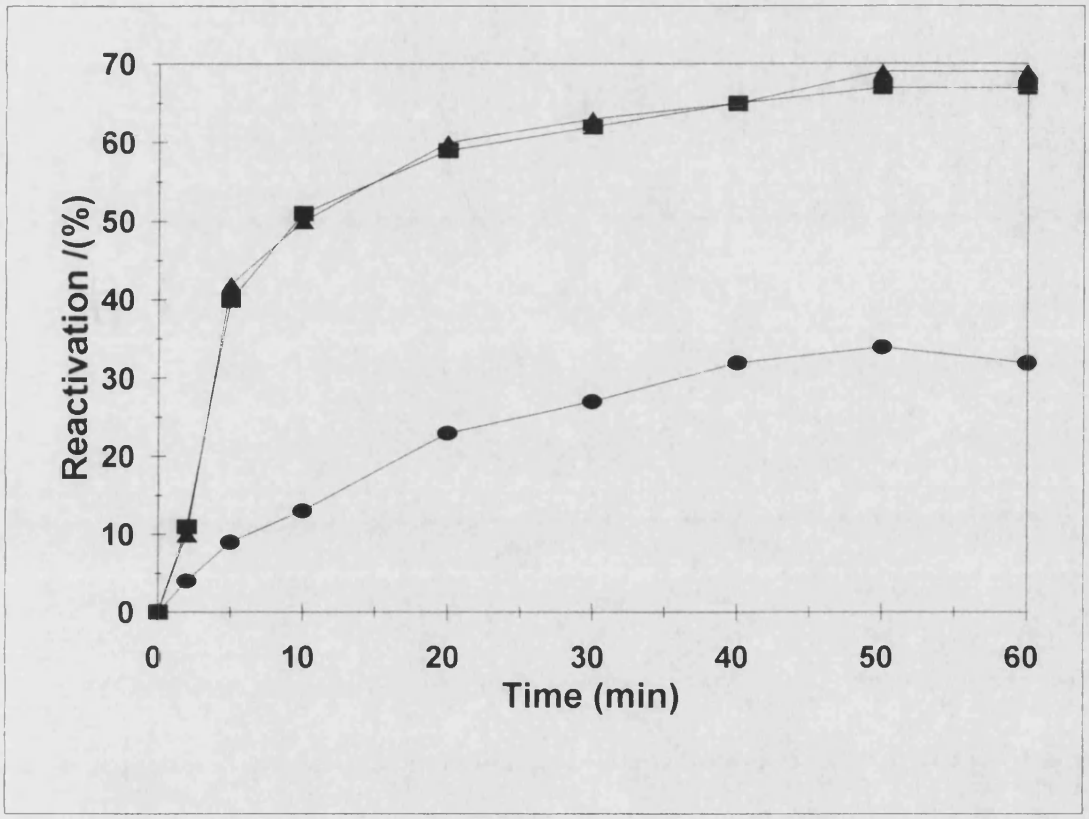

Figure 6.4. The effect of temperature on the refolding of denatured and reduced lysozyme

Lysozyme was reduced and denatured in 6 M GuHCl (see Figure 6.3) Renaturation was performed at 10 °C (●), 40 °C (■) and 60 °C (▲). Enzyme activity was assayed at several time points and expressed relative to native lysozyme at the same concentration.

6.1.3.2 The effect of protein concentration on refolding

Another important parameter in the refolding of proteins is the protein concentration. The detrimental effect of high protein concentrations on the corresponding recovery of activity is observed. Figure 6.5 shows the reactivation of denatured and reduced lysozyme as a function of protein concentration. The recovery of activity by refolding lysozyme in aqueous solutions is found to be optimum at low protein concentrations. As the protein concentration is increased from 0.015 mg/ml-0.2 mg/ml the corresponding recovery of activity is significantly reduced. After 30 min the reactivation of lysozyme appears to be completed since only very slight increase in activity occur after this time point. At 30 min the protein is reactivated to 60% at 0.015 mg/ml, 50% at 0.05 mg/ml, 35% at 0.1 mg/ml and 15% at 0.2 mg/ml. Another important observation is aggregation formation as measured by A_{450}. As the protein concentration was increased for refolding the A_{450} readings also increased due to the scattering of these protein aggreagtes (results not shown).

The decrease in the refolding yield at high protein concentrations has been explained by the kinetic competition between folding and aggregation reactions as mentioned in Chapter 2. Ideally renaturation procedures should be performed at high protein concentrations. However, this leads to a greater chance for the proteins to aggregate. When proteins are unfolded, their hydrophobic cores are exposed to the solvent and aggregation results form the non-specific interactions between hydrophobic regions of different protein molecules. After renaturation of lysozyme (Figure 6.5) at different protein concentrations, A_{450} measurements showed increases in light scattering as protein concentration used for refolding increased confirming that aggregation was the major cause for reduced activity.

It is widely accepted that the major problem in unfolding/refolding experiments is the kinetic competition between folding and aggregation. This is due to the synchronous action of intra- and intermolecular interactions in the folding reaction:

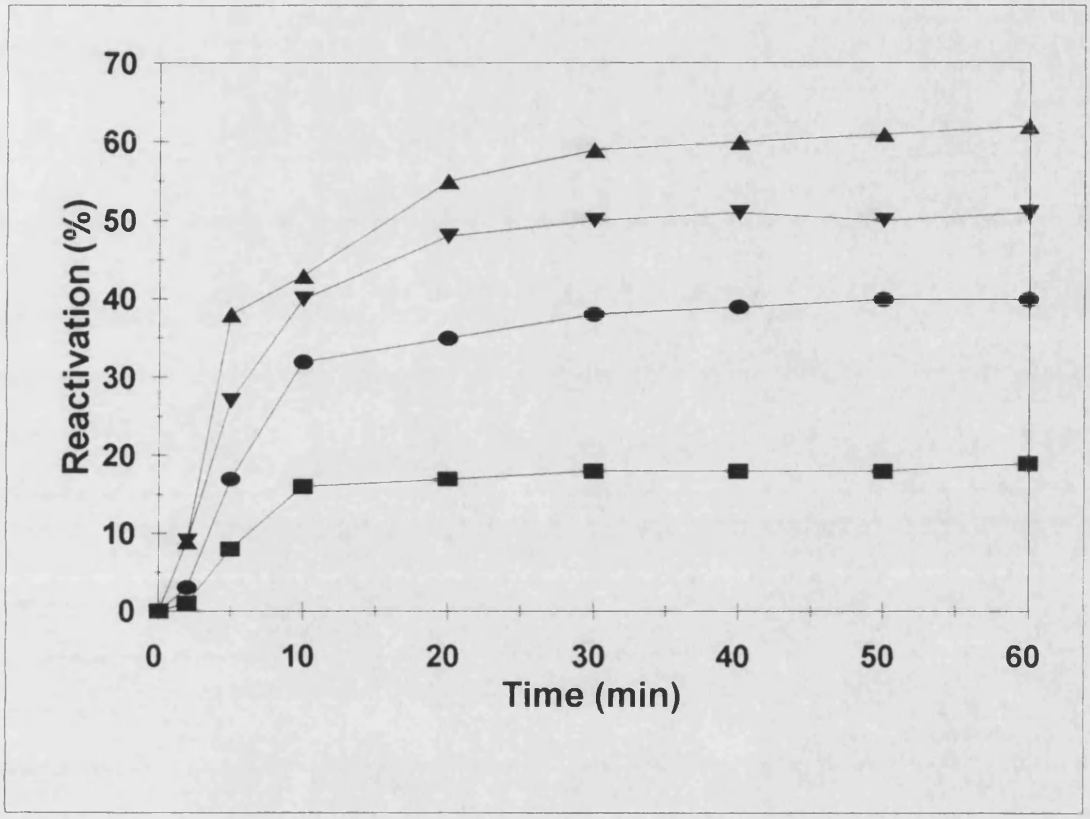

Figure 6.5. The effect of protein concentration on the refolding of denatured and reduced lysozyme

Renaturation-reoxidation of the enzyme solution was initiated by adding denatured and reduced protein to different final concentrations, 0.015 mg/ml (▲), 0.05 mg/ml (▼), 0.1 mg/ml (●) and 0.2 mg/ml (■) to the renaturation buffer (0.1 M Tris-HCl, pH 8.2, 1 mM EDTA, 3 mM reduced glutathione and 0.3 mM oxidised glutathione), mixing with a vortex mixer for 15 s and incubating at 40°C. Enzyme activity was assayed at several points and expressed relative to native lysozyme measured at the same concentration.

$$N \Leftrightarrow D \Rightarrow \text{Aggregates} \qquad (6.1)$$

where N and D are the native and denatured forms of the protein respectively. Lowering the protein concentration increases the yield of activated protein by eliminating the irreversible aggregation reaction. The best renaturation conditions are therefore those that optimize refolding and minimize aggregation.

6.1.4 Summary

The results obtained in this section have described the refolding of lysozyme in an aqueous environment and some of the problems encountered. Data presented has shown that lysozyme can be inactivated at very low GuHCl concentrations. These low denaturing conditions cause no major conformational change and inactivation occurs due to ionic strength effects.

Denatured, non-reduced lysozyme with its disulphide bonds intact can be completely refolded into its three-dimensional structure. The presence of the disulphide bonds restricts the protein structure to have a more random coil structure. By dilution of the denatured molecule into refolding buffer the protein returns to its native state.

Reduction of the protein allows the protein to have a completely unfolded randomly coiled configuration in 6 M GuHCl. Refolding now becomes more complicated, since the disulphide bonds need to be correctly reformed. Results presented have shown that only 65% activity can be achieved under optimum conditions. Temperature was shown to effect the rate of reactivation. A decrease in temperature to 10°C reduced the activity gained by 50%. However, an increase in temperture to 60°C did not improve the yield of reactivation of reduced lysozyme compared to that at 40°C. Protein concentration was also shown to effect the yield of reactivation of native lysozyme. Increases in the initial lysozyme concentration decreased the rate of reactivation. This has been explained by the formation of aggregates at higher protein concentrations in a competing

reaction. The light scattering observed at A_{450} confirmed that aggregation was the side reaction that dominated the refolding reaction.

6.2 Backward transfer of native lysozyme from reversed micelles

Conditions were investigated in Chapter 4 for the forward transfer of lysozyme into reversed micelles. Now that the conditions have been established to achieve maximum transfer of lysozyme into AOT/isooctane micelles experiments need to be performed to re-extract the protein into a fresh aqueous solution and to determine its activity. This is another important step in the overall procedure since once the protein is refolded in the micellar environment, suitable re-extraction methods must be developed in order to recover the protein. Unfortunately, there has been a poor methodology developed for the backward transfer process in comparison with the forward transfer process (see Chapter 2). It is important to recover the protein back under conditions that will not perturb the tertiary structure of the protein. Two methods were used, the first utilises a combination of ionic strength and pH and the second uses a combination of ionic strength and ethyl acetate. A third novel method based on isopropyl alcohol was also attempted.

6.2.1 The effect of pH on the backward transfer of lysozyme

The effect of pH on the backward transfer of lysozyme was studied. To recover lysozyme from a micellar phase into a fresh aqueous phase it could be possible to use the conditions opposite to those which would facilitate those of the forward transfer. Figure 6.6 illustrates that between pH 4-10 very little protein is transferred back into the aqueous phase. At a pH value of about 4 less than 10% of the protein is transferred into the organic phase and even at pH 10.0 only 20% transfer occurs. However, as the pH of the solution rises towards the pI of lysozyme (10.9) the percentage recovery increases greatly with maximum protein transfer occurring between pH 11-11.2 (90%) and this decreases to 60% at a pH

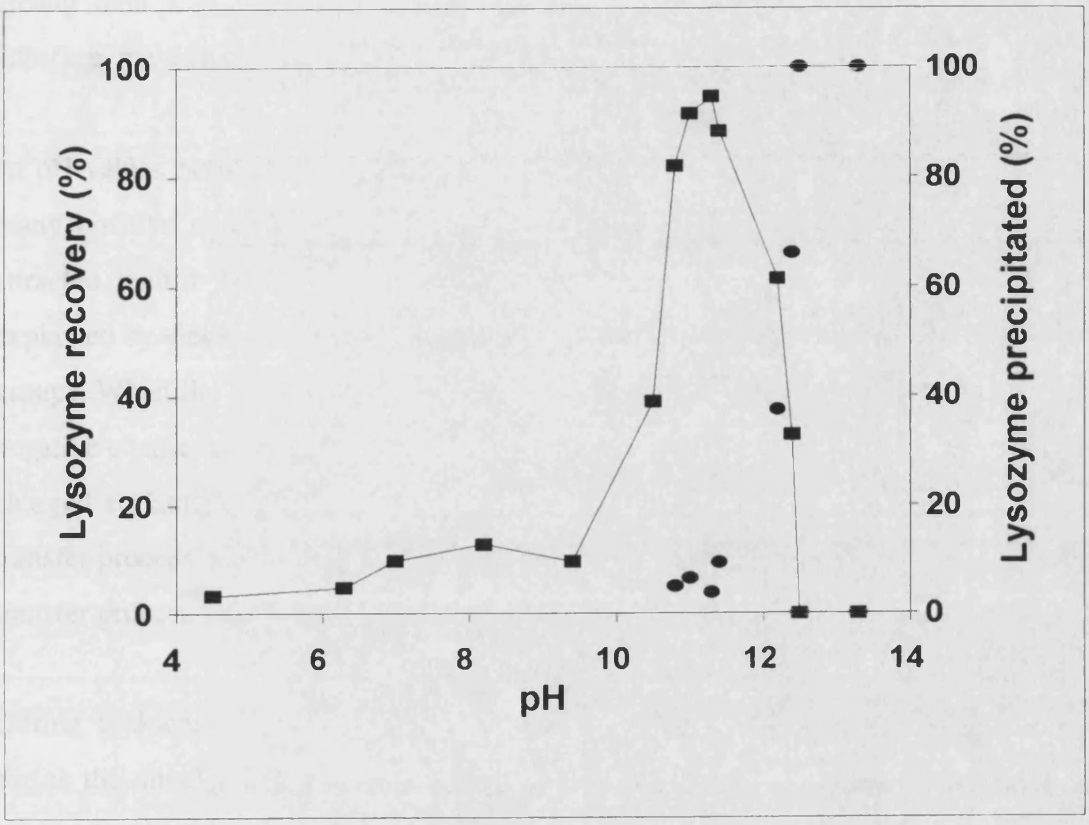

Figure 6.6. Back transfer of native lysozyme from 50 mM AOT/isooctane micelles using a combination of high ionic strength at various pH for 30 min

50 mM AOT/isooctane micelles containing 1 mg/ml lysozyme from the forward transfer were mixed with equal volumes (4 ml) of an aqueous phase containing 1 M KCl at a variety of pH values (4-13) for 30 min. The mass of the protein which precipitated at the interface (●) and recovered in the aqueous phase (■) was quantified.

value of 12.2. Figure 6.6 also shows the amount of precipitate formed at the interface as a function of pH. No precipitate was formed between pH 4 to 10 and about 10% precipitate was formed at the aqueous/organic interface at pH 11.0. This was observed to rise to about 40% at a pH of 12.2 and 100% at pH values greater than 13.0. The mass balance between the organic, aqueous phase and interface gave an error of ± 5%.

At pH values between 4-10, well below the isoelectric point of lysozyme (10.9), many positive charges are carried on the surface of lysozyme thus it remains attracted within the micelle due to these favourable interactions. This can be explained by electrostatic interactions between the protein and the surfactant head groups. When the pH is higher than the pI of the protein, the protein carries a net negative charge and is forced out of the micelle due to repulsion by the negatively charged surfactant head groups. However, it is interesting to note that the back transfer process occurs over a very narrow pH range in comparison to the forward transfer process which takes place over about 5 pH units.

During backward transfer, a high salt (KCl) concentration (1 M) is required to shrink the micelle and expell the protein out. By using a high ionic strength the positively charged potassium ions neutralise the negatively charged surfactant head groups thus screening the interactions between each other resulting in smaller micelles. The micelles therefore become too small to accommodate the larger protein molecule (Section 4.3.1.1). Kinugasa and Watanabe (1992) have performed the backward transfer of lysozyme under similar conditions and their results are comparable to those obtained above. However, there is no mention of the formation of a precipitate at the interface, especially when the pH of the medium exceeds 12.

The effect of pH on the backward transfer of lysozyme was also investigated over a longer contact time (48 minutes). Figure 6.7 shows that time plays a significant role on the protein/surfactant complex and in which phase the protein is found in. As the aqueous phase pH increases from 3-13 the transfer of lysozyme into the

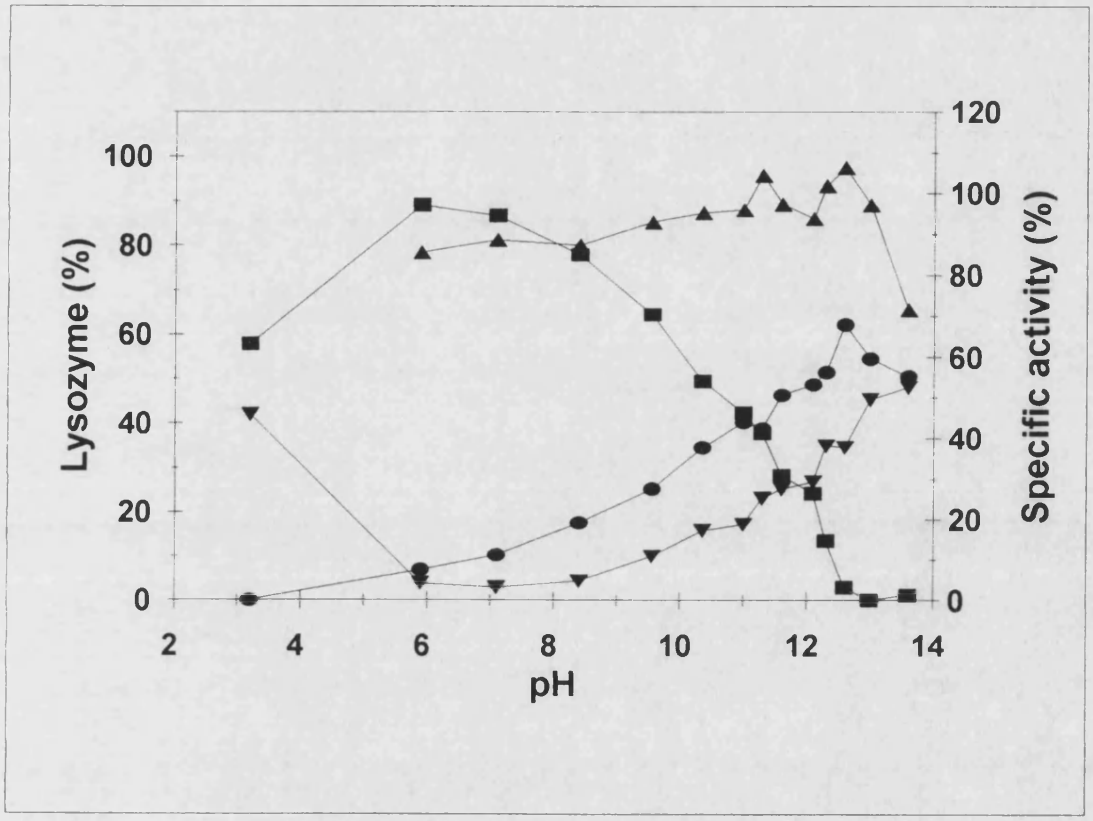

Figure 6.7. Back transfer of native lysozyme from 50 mM AOT/isooctane micelles using a combination of high ionic strength at various pH for 48 min

The back extraction was performed as in Figure 5.6. Protein content in both the organic phase (■)and aqueous phase (●) was measured after phase separation. The amount of precipitate (▼) formed could be seen visually by the eye and was determined by a simple mass balance. Activity (▲) measurements relative to that of native lysozyme were determined in the aqueous phase after the back extraction. The backward transfer was performed for 48 minutes.

aqueous phase increases giving a maximum recovery of 62% at a pH of 12.6. This shows that a prolonged mixing time has a detrimental effect on protein transfer. In the previous experiment a mixing time of 30 minutes was used and at pH values of 12-13 the protein recovered in the aqueous phase was 80%. Another distinct factor that was observed visually was the increase in the amount of interfacial precipitate formed as the pH rose from 10-13.6. A simple mass balance indicated that all the protein had been extracted from the organic phase using a salt concentration of 1 M KCl and pH values of 12.6 or greater. However, there is also a significant increase in precipitate formation and loss of protein activity. The backward transfer step was optimized by using the minimum time for back transfer to be achieved (30 minutes) and a pH value of between 11-11.4 to minimize protein precipitation and denaturation effects.

6.2.1.1 Structure of lysozyme measured by near-uv CD after the backward transfer

The spectrum of lysozyme (1 mg/ml) after it was back transferred from the micelles using 1 M KCl at a pH of 11.2 giving 90% recovery in the aqueous phase is shown in Figure 6.8. The spectrum for native lysozyme and that of the transfer into the micelle are also shown. It is interesting to note that by returning the protein back into the aqueous environment the tertiary structure returns with the resulting and the rest of the spectrum almost identical to the native protein in the aqueous environment before the transfer. The protein also retained most of its activity (~ 98%) after this back extraction step. Obviously the protein unfolding that takes place in the micelles is reversible since the tertiary structure is recovered together with most of the activity. However, since the tertiary structure is lost, this does not necessarily mean that the protein is fully unfolded since secondary structure may still be present.

Steinmann *et al.* (1986) studied the conformation of lysozyme by CD in the near-uv in AOT/isooctane reversed micelles. Their CD data showed that lysozyme was irreversibly denatured. The results presented show that when lysozyme was

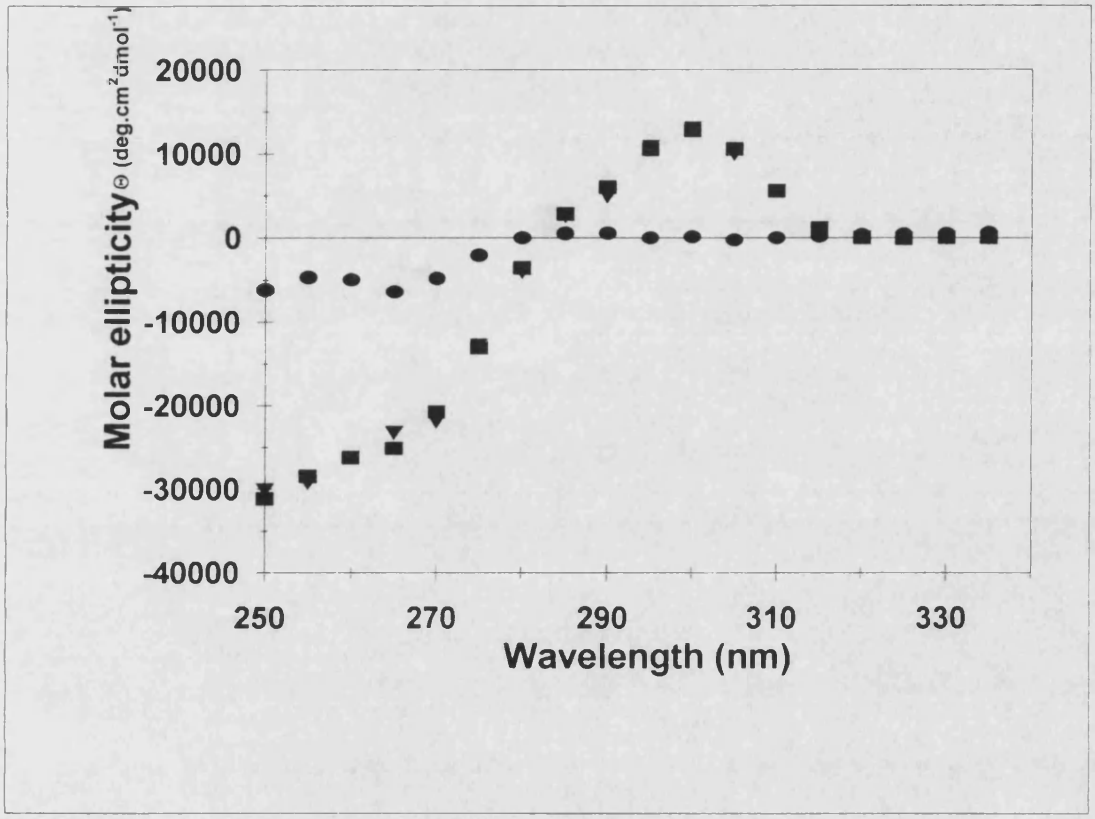

Figure 6.8. Circular dichroism spectra of lysozyme after backward transfer from 50 mM AOT/isooctane micelles

Circular dichroism spectra in the near-uv were recorded for lysozyme in the aqueous environment (■) and inside 50 mM AOT/isooctane micelles (●). The spectrum of native lysozyme (▼) after the backward transfer was also recorded. The samples were left for 30 min before scanning between 250-320 nm. The spectra were recorded at a scanning rate of 2 nm/min and are an average of 3 determinations after subtraction of the buffer blanks.

recovered, it retained most of its activity and the tertiary structure was recovered which contradicts the hypothesis of irreversible denaturation. A possible explanation is that in the experiments performed above, the protein was back extracted within 10 min which was probably too short a time for irreversible denaturation to occur. The time that the protein is left in this particular micellar system may also be of great importance according to the results presented. Unfortunately, due to the unavailability of the substrates used by Steinmann *et al.* (1986), no activity measurements could be recorded in the micellar environment and no time dependence studies could be performed. However, the experiments performed allowed complete recovery of the protein and therefore the AOT has a reversible denaturing effect on lysozyme.

Recovering the protein back into a fresh aqueous solution using high pH (11.2) and KCl (1 M) enables the protein to return to its native tertiary structure and the CD spectum is identical to the one before the forward transfer. Activity measurements of the recovered protein found that 98% activity was regained. Thus, the denaturation that took place inside the micelle is a reversible process. The reversibility of this denaturation is due to the short time length of the experiments performed. A contact time of 15 min was used for the forward transfer then the protein was immediately back transferred for 30 min, preventing the protein from being exposed to the micellar environment for too long. This allows the protein to be in the vicinity of the micellar environment for a very short period and prevents the protein from being irreversibly denatured by the AOT head groups.

6.2.2 The effect of ethyl acetate

Figure 6.9 shows that it is possible to back extract native lysozyme (1 mg/ml) from reversed micelles using a combination of high ionic strength to shrink the micelles and ethyl acetate to disrupt the micelles, Hagen (1989). In the absence of ethyl acetate only 30% of the protein is back extracted but as the amount of ethyl acetate is increased to 0.4 ml/ml solution, 80% of the protein is back extracted.

Between 0.4-1 ml ethyl acetate/ml solution there is no further increase in the transfer of protein into the aqueous phase and between 1.1-1.3 ml ethyl acetate/ml solution the backtransfer process begins to decline. Figure 6.9 also shows that the protein left in the organic phase after the back extraction falls to below 10% after 0.6 ml ethyl acetate/ml solution. A small amount of precipitate was seen at the interface below an ethyl acetate concentration of 1 ml/ml solution but this was generally less than 5%. Between 1-1.3 ml/ml solution the interfacial precipitate began to increase to between 10-15%. The mass balance of the protein between the organic and aqueous phases gave an error of less than 5%. Over the ethyl acetate concentration range 0-1 ml/ml solution lysozyme retained all of its activity in the resulting aqueous solution after the backward transfer. However, between 1-1.3 ml ethyl acetate/ml solution the activity begins to decrease slightly as the interfacial precipitate is observed to increase.

Hagen (1989) has found that increasing ethyl acetate from 0-0.18 ml ethyl acetate/ml solution the amount of RNase back extracted increased fourfold. Since ethyl acetate can have a denaturing effect on proteins Hagen used a volume of 0.1 ml ethyl acetate/ml solution giving an 80% transfer of protein. Woll *et al.* (1987) completely recovered RNase and concanavalin A from AOT/isooctane reversed micelles by using ethyl acetate but could not use pH and ionic strength to back extract the proteins. Ethyl acetate is a polar molecule and is able to disrupt the micellar environment. Together with the high salt concentration used (1 M KCl) which shrinks the micelles, lysozyme was forced out of the micelles and recovered in the aqueous phase.

The limited number of backward transfer methods available show that they are protein specific and no generalisation can be made for a particular protein. Three methods for the backward transfer of lysozyme from a 50 mM AOT/isooctane reversed micellar phase have been attempted. The first one uses a combination of high salt (KCl) and a high pH (11.0) has been successfully used giving an 80% backward transfer with the protein retaining 100% activity. At pH values above the pI of lysozyme (10.9) the electrostatic interactions between the protein and

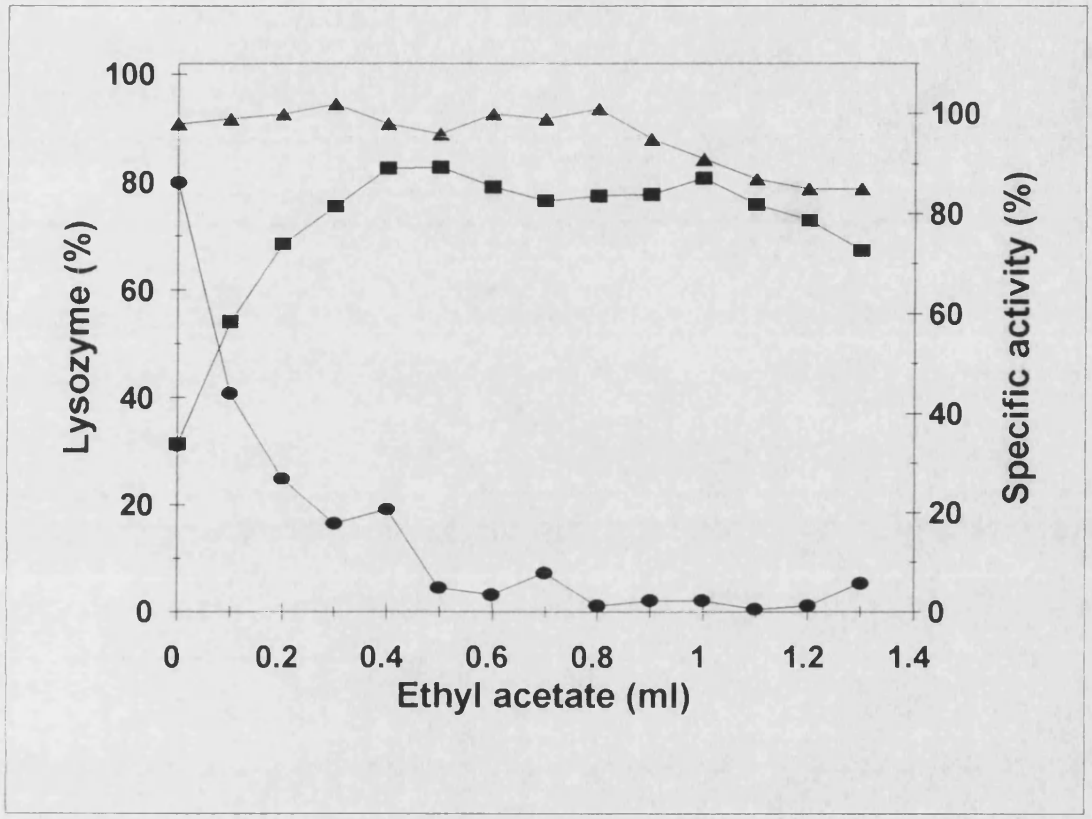

Figure 6.9. Back transfer of native lysozyme from 50 mM AOT/isooctane micelles using a combination of high ionic strength and ethyl acetate

The back extraction process was performed as previously (Figure 6.8) to which various amounts of ethyl acetate were added (0-1300 µl). The samples were mixed for 30 min and then centrifuged for 10 min at 2000 rpm. This gave two clear phases after which protein concentration was determined in the aqueous (■) and organic (●) phases using A_{280} measurements. The activity (▲) relative to that of native lysozyme was also monitored.

the micellar interface cause the protein to be repelled out of the aqueous core. In addition the high salt concentration causes the micelle to shrink in size which also helps the protein to be size-excluded from the micelle. The ability to recover a protein from a reversed micellar solution and for the activity to remain after the recovery, are functions of the proteins specific structure. In some cases it is possible that certain proteins solubilised in micelles could form strong specific interactions between certain amino acid side chains and the micellar interface thus making recovery more difficult.

The second method using ethyl acetate and high KCl (1 M) concentration allowed 80% of the protein to be recovered whilst retaining all its activity. The polar organic solvent disrupts the micelle and together with the shrinking of the micelle caused by the KCl concentration the protein is forced out. However, high concentrations of ethyl acetate resulted in a loss in the activity of lysozyme (10-15%) together with the formation of an interfacial precipitate.

6.2.3 The effect of contact time

In Section 4.2.2, it was found that the lysozyme was transferred into reversed micelles very quickly (less than 5 min) and this process can be 10-1000 times faster than the backward transfer. Similarly experiments were performed on the recovery of the protein from the reversed micellar phase into a fresh aqueous phase. Figure 6.10 shows the protein leaving the organic phase and being recovered in the aqueous phase over a time period of 60 minutes. It should be noted that under the conditions employed in this process (pH 11.2 and 1 M KCl) no precipitate was formed at the interface as these were the optimum conditions for the backward transfer process, Section 6.2.1. The mass balance of the protein between the organic and aqueous phase gave an error of less than 5%. Figure 6.10 shows that after a time period of 5 min, 10% of the protein was recovered in the aqueous phase. After 20 min, 50% was recovered and after 30 min 90% of the protein was re-extracted into the aqueous phase. Between 30-60 min no further increase in the recovery of protein occurred.

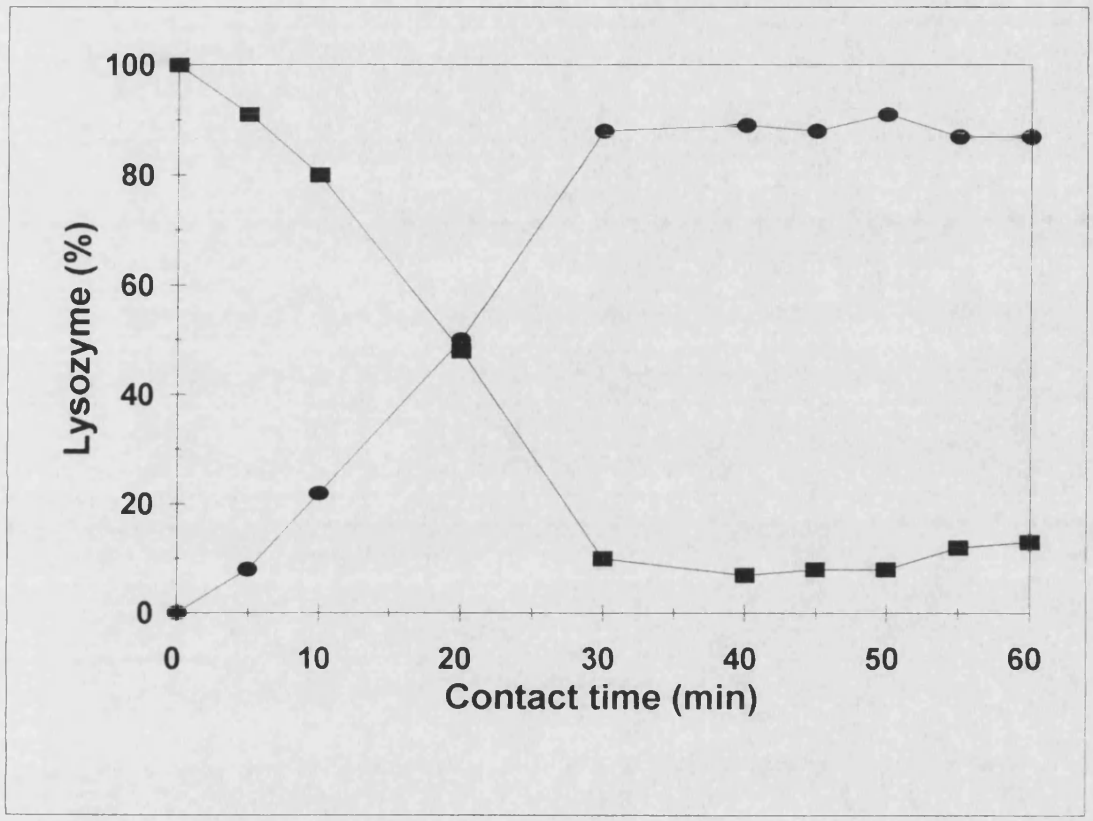

Figure 6.10. The effect of contact time on the backward transfer

The backward extraction of lysozyme involved mixing 4 ml of the organic phase containing 1 mg/ml of lysozyme with an aqueous phase (1 M KCl in 25 mM phosphate buffer, pH 11.2) for various (0-60 min) times. The phases separated quickly yielding two clear phases and protein concentration was determined in the organic (■) and aqueous (●) phases using A_{280} measurements.

Thus, the minimum time required for the protein to be back extracted is 30 min whereas for the forward extraction only 1 min was required. The backward transfer process is therefore 30 times slower and must occur via a different mechanism. In the forward transfer process the protein diffuses towards the aqueous/organic interface, which is a monolayer of surfactant, and forms a micelle around itself by carrying water and surfactant into the oil phase. However, for the reverse process the protein filled micelle must approach the interface and collide with it causing the protein and water to be reabsorbed into the aqueous phase whilst the surfactant adsorbs back to the interface. Kinugasa *et al.* (1991) have suggested that the backward transfer process is determined by an interfacial process and the rate limiting step is the coalescence and the merging of a protein filled reversed micelle with the interface.

6.2.4 Summary

In order to recover the protein form the reversed micellar phase, two methods have been successfully used. The first method of using pH and 1 M KCl allowed the protein to be recovered under conditions opposite to those which facilitate the forward transfer. At pH values far and below the isoelectric point of lysozyme, the protein would have a high density of positive charges and favourable electrostatic interactions between the surfactant interface and protein would retain the protein in the aqueous core of the micelle. As the pH increases towards the pI, protein recovery steadily increases and becomes optimum just after the pI. The protein now carries a net negative charge and can be expelled out of the micelle due to the repulsion between the micellar interface and the protein. The high KCl (1 M) concentration also helps with the recovery process by shrinking the micelle, thus size excluding the protein from the aqueous core. The protein retained all of its activity after this backward extraction showing that the method had no detrimental effects on the protein.

Another important parameter in this study was the contact time used for the backward transfer process. Results show that a longer mixing time of 48 min compared to 30 min causes less protein to be recovered and a protein/surfactant

complex is formed at the interface. This is important since the contact time can be optimised to obtain better recovery and less formation of a precipitate at the interface. It is believed that a longer mixing time can lead to protein denaturation and hence unfolding of the molecule. This leads to binding of surfactant to the denatured protein which yields a loss in recovery. It was also noted that the optimum recovery of lysozyme from the micellar phase occurred over a very narrow pH range (11.0-11.5) compared to the yields transferred in the forward transfer. Also using pH values greater than 13.0 showed no recovery of the protein and a large precipitate was seen at the interface. At these high pH values, lysozyme is believed to denature and unfold, again forming a protein/surfactant complex.

The second approach used for the recovery of the protein from the micellar phase used a combination of ethyl acetae and high salt (KCl). The polar molecule is believed to disrupt the micelle and together with the shrinking caused by the KCl, extracts the protein back into the aqueous phase. Again the protein retained all of its activity. However, care needs to be taken in the amount of ethyl acetate used since it can have denaturing affects on the protein.

6.3 Refolding lysozyme in a reversed micellar environment

Conditions have now been investigated for refolding denatured and reduced lysozyme in an aqueous environment with an optimum recovery of activity of 65%. It is believed that the refolding problem lies not in the formation of the disulphide bonds, since the Ellman assay used showed that all four disulphides bonds were formed. The major problem of refolding lies in the fact that the protein concentration has be very low in order to prevent the competing aggregation reaction. Even at extremely low concentrations, only 65% activity could be regained for lysozyme.

Reversed micelles have been used to investigate the problem of refolding in order to reduce the competing reaction of aggregation. Under certain conditions,

denatured protein molecules were transferred individually into the aqueous cores of micelles. This should allow the protein to refold in its own separate environment avoiding the problem of aggregation. The other advantage of using reversed micelles is that the protein concentration used can be as high as 1 mg/ml which is much higher than used at present.

6.3.1 Refolding non-reduced lysozyme

Conditions were investigated in Section 4.3 for the transfer of non-reduced lysozyme into AOT/isooctane reversed micelles. This section presents results on the refolding of non-reduced lysozyme in GuHCl and mixtures of GuHCl and urea.

6.3.1.1 Lysozyme (non-reduced) in GuHCl

The backward transfer of lysozyme in GuHCl was performed using the same method as used previously for the native protein (1 M KCl at a pH of 11.2) (Section 6.2.1). After the back extraction most of the protein (90-100%) was recovered and found to contain 100% activity (Figure 6.11). The aqueous core of the reversed micelles contain less than 1% water thus, on returning the lysozyme back in to a fresh aqueous solution dilutes the denaturant significantly. Since the protein is in the non-reduced form and the disulphide bonds are intact, the release of the protein from the reversed micelle phase into an aqueous phase results in a 100 fold dilution of the GuHCl concentration and this enables the protein to return to its native conformation. At these low denaturant concentrations the lysozyme molecule is in a near-native state and the back extraction merely washes the GuHCl from the protein allowing activity to be regained.

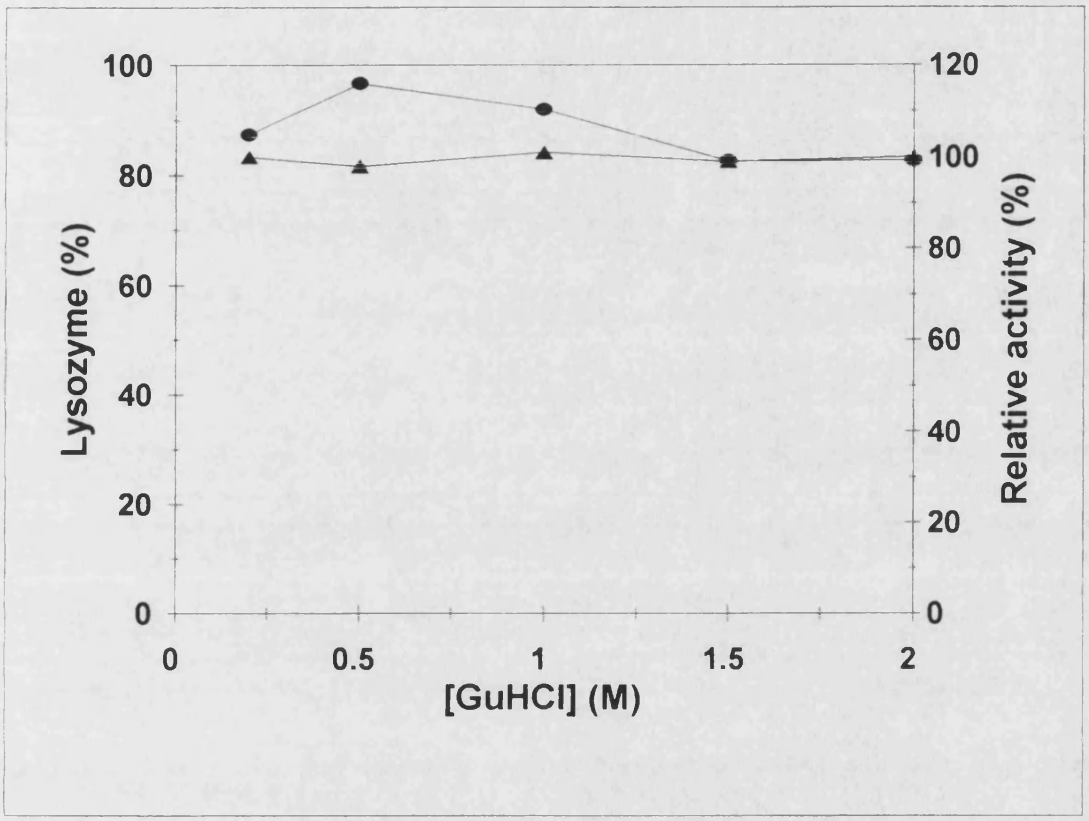

Figure 6.11. The backward transfer of lysozyme (non-reduced) in GuHCl from 50 mM AOT/isooctane reversed micelles

The forward extraction of lysozyme involved mixing equal volumes (5 ml) of 50 mM AOT/isooctane with an aqueous phase (1 mg/ml protein in 0.1 M Tris-HCl buffer containing 0.2-2 M GuHCl) for 15 min. 4 ml of the protein containing organic phase was then contacted with the same volume of a 1 M KCl solution in Tris-HCl, pH 11.2 for 30 min. Protein concentration was determined in both phases and the amount of back transfer (●) calculated. Activity (▲) of the recovered protein was also measured.

Over the range of GuHCl concentration (0.2-2 M) studied it was found that between 90 and 100% of the protein is recovered in the resulting aqueous phase. At these relatively low concentrations of GuHCl it may be expected that the lysozyme has a near-native like structure due to the presence of the disulphide bonds in the protein. Hamaguchi and Kurono (1963) have monitored the structural transitions by circular dichroism that occur when lysozyme is denatured in GuHCl. They found that there are no structural changes in lysozyme using concentrations of up to 3.2 M GuHCl but above this concentration a transition in the structure occurs which is complete at 5 M. These results are similar to the results presented in Secction 4.4.3. Thus, in the GuHCl concentration range used in Figure 6.11 there is very little change occurring in the overall structure of the protein.

6.3.1.2 Lysozyme (non-reduced) in mixtures of GuHCl and urea

Mixtures of the denaturants GuHCl and urea at various ratios have been found to significantly improve the transfer process at higher denaturant concentrations. The next step is to determine the conditions under which the reverse process of back extraction takes place. The two backward transfer methods used successfully in Section 6.2 i.e. high pH and ethyl acetate were used.

The back transfer of non-reduced lysozyme using a high pH from two systems (GuHCl:urea (0.5:4.5 and 1:1)) are shown in Figure 6.12. In both systems lysozyme is returned into the aqueous phase (90-100%) with very little or no protein remaining in the organic phase. Activity measurements of the aqueous phase showed the protein to regain 100% of its activity. Therefore, the backward extraction step which uses 1 M KCl and a high pH (11.2) can be used effectively with these mixed denaturant systems.

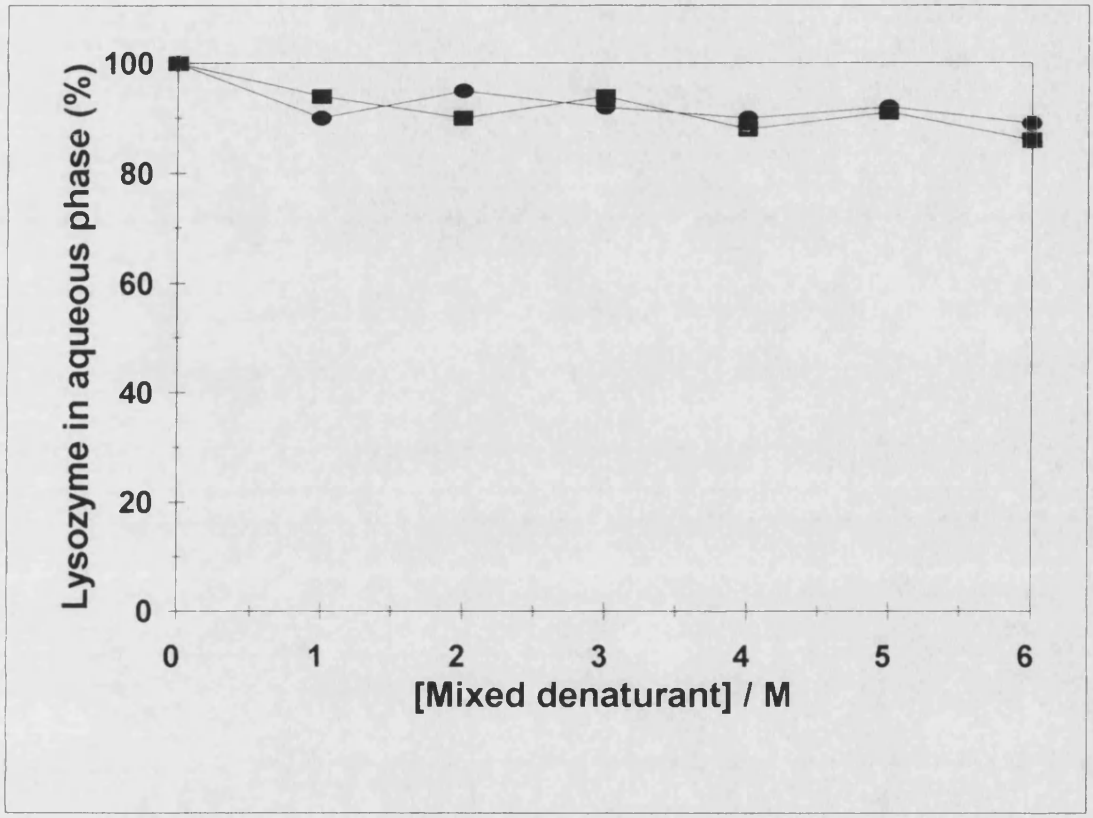

Figure 6.12. The backward transfer of non-reduced lysozyme from 50 mM AOT/isooctane micelles using a high pH (11.2) after forward transfer using mixed denaturants

The backward transfer was performed by mixing 4 ml of the organic phase containing the protein from the forward transfer (GuHCl:urea (1:1) ● and (0.5:4.5) ■) with 4 mls of an aqueous phase consisting of 1 M KCl in Tris-HCl, pH 11.2 for 30 min. The samples were then centrifuged at 2000 rpm for 10 min and protein concentration was determined in each phase.

Transferring the protein from the reversed micellar phase (containing 1-2% water) to the fresh aqueous phase dilutes the denaturant concentration inside the micelle about 100 times and returns the protein back to its native conditions. Since the protein is in the non-reduced form and its disulphide bonds remain intact, no reoxidation is required.

Figure 6.13 shows the backward transfer of non-reduced lysozyme from two mixed denaturant systems GuHCl:urea (0.2:4.8 and 0.3:4.7) using ethyl acetate. The back extraction improves as the ethyl acetate concentration is increased with 35% transfer of protein in the absence of ethyl acetate and 65% transfer at 100 µl ethyl aceteate/ml solution. Between 100-300 µl ethyl acetate/ml solution there is no further change in the backward transfer and in the range 300-400 µl ethyl acetate/ml solution the transfer of protein begins to decline. The extraction curves obtained for both of the systems were similar. In both cases the protein was able to be back extracted while retaining all of its activity on returning to the aqueous solution. However, at higher ethyl acetate concentrations (> 300µl/ml) 10-15% of the activity was lost in the aqueous phase.

Results have been presented in Chapter 4 on the denatured, non-reduced form of lysozyme. The protein was partitioned into reversed micelles at high concentrations of mixed denaturants. However, unfolding did not occur to the same extent as with GuHCl and this was confirmed by conformational studies. Backward transfer of this denatured protein from the micelles resulted in 100% activity in the aqueous solution. This was explained by the fact that the dilution factor was about 100 fold when passing from the micellar aqueous core to the aqueous solution. This process could be used for non-disulphide bond containing proteins provided the protein studied could be transferred in the fully unfolded form.

Both the backward transfer procedures using high pH and ethyl acetate, returned the protein back to its native structure as 100% activity was regained. The CD spectra was recorded and found to be indistinguishable from the native protein.

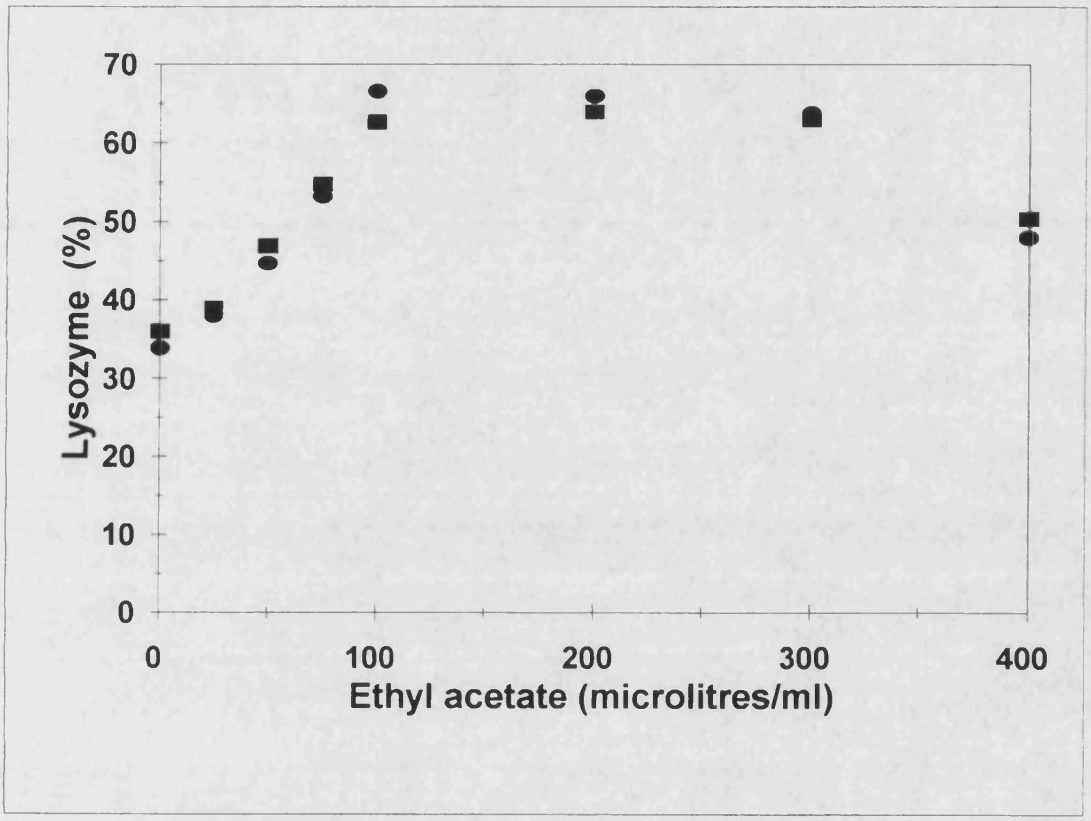

Figure 6.13. The backward transfer of non-reduced lysozyme from 50 mM AOT/isooctane micelles using ethyl acetate after the forward transfer using mixed denaturants

The backward transfer was performed by mixing 4ml of the organic phase containing the protein from the forward transfer (GuHCl:urea (0.2:4.8) ■ and (0.3:4.7) ●) with 4ml of an aqueous phase containing 1 M KCl in Tris-HCl, pH 8.6 with different amounts of ethyl acetate for 30 min. The samples were then centrifuged at 2000 rpm for 10 min and protein concentration determined in the aqueous phase using A_{280} measurements.

Therefore, the back extraction step enables the protein to return to the aqueous environment, removing the presence of denaturant, and returning the protein to its native environment. However, the ethyl acetate method only recovered 65% of the protein in comparison with 90-100 % when conditions of 1 M KCl at pH 11.2 were used.These results show that there may be a potential application for the refolding of non-disulphide containing proteins such as carbonic anhydrase if they could be transferred into micelles (possibly non-ionic in nature). The micelles could be used to isolate the protein molecules and thereby prevent aggregation. A back extraction step could then be utilized to return the protein into an aqueous environment diluting the denaturant concentration and returning the protein to its native state. This simple back extraction step would remove the large volumes required for refolding on a large scale. The backward transfer process returned 90% of the lysozyme present in the organic phase, from the forward transfer, over the GuHCl concentrations studied and allowed full regain of its activity.

6.4 Refolding reduced lysozyme

Since the conditions were investigated in Chapter 4 for the transfer of reduced lysozyme in GuHCl and mixtures of GuHCl and urea, it becomes important to see if the protein can be back extracted into a fresh aqueous solution. This section shows results for both GuHCl and mixtures of GuHCl and urea.

6.4.1 Removal of denaturant via dilution

The transfer of denatured and reduced lysozyme into AOT/isooctane reversed micelles has already been investigated in Chapter 4. After this transfer step the protein needs to be correctly folded into its native state before it can be re-extracted into the aqueous environment.

In order to return the protein to a native environment, dilutions need to be performed to reduce the denaturant and DTT concentrations to a minimum to enable the protein to refold. All the refolding experiments performed in this

chapter were attempted after dilution of the denaturant and DTT inside the micelles.

6.4.1.1 Monitoring micelle size using Karl-Fischer titration

The dilutions of the denaturant inside the micelles was monitored by measuring the increase in water content inside the micelles by Karl-Fisher titration which gives one an indication of the dilution factor as a result. Since it is difficult to know exactly what concentration of denaturant is present in the micelles after the initial forward transfer it then becomes difficult to know how much of the denaturant is left inside the micelle after the dilutition. However, the dilution factor of the denaturant inside the micelles can be calculated using Karl-Fisher titration. Eicke *et al.* (1976) showed that a dynamic equilibrium exists in reversed micellar solutions where micelles can collide with each other and exchange there solutes.

Initially, lysozyme (0.5 mg/ml) was denatured and reduced in 2 M GuHCl and then transferred into 50 mM AOT/isoctane micelles. Figure 6.14 shows how the number of dilutions performed affects the water content in the micelles. After each dilution the size of the reversed micelles was found to increase. The size of the micelles increase from a Wo value of 5.9 to 10.5 by the fourth dilution step using 0.1 M KCl. After the fourth dilution the buffer was changed to 0.1 M NaCl. This causes the micelles to increase in size and results in an increased dilution of the denaturant inside the micelles. This causes the value of Wo to increase from 10.5 at the 4th dilution to 42 at the 7th dilution. The size of micelles formed using different salts has been investigated earlier in the thesis (Section 4.3.1.1). Sodium ions produce larger micelles than potassium ions since they are smaller in size which effectively means that they neutralise the negatively charged surfactant head groups to a lesser extent than the larger potassium ions. Also sodium ions have a greater water of hydration present which also increases the size of micelles.

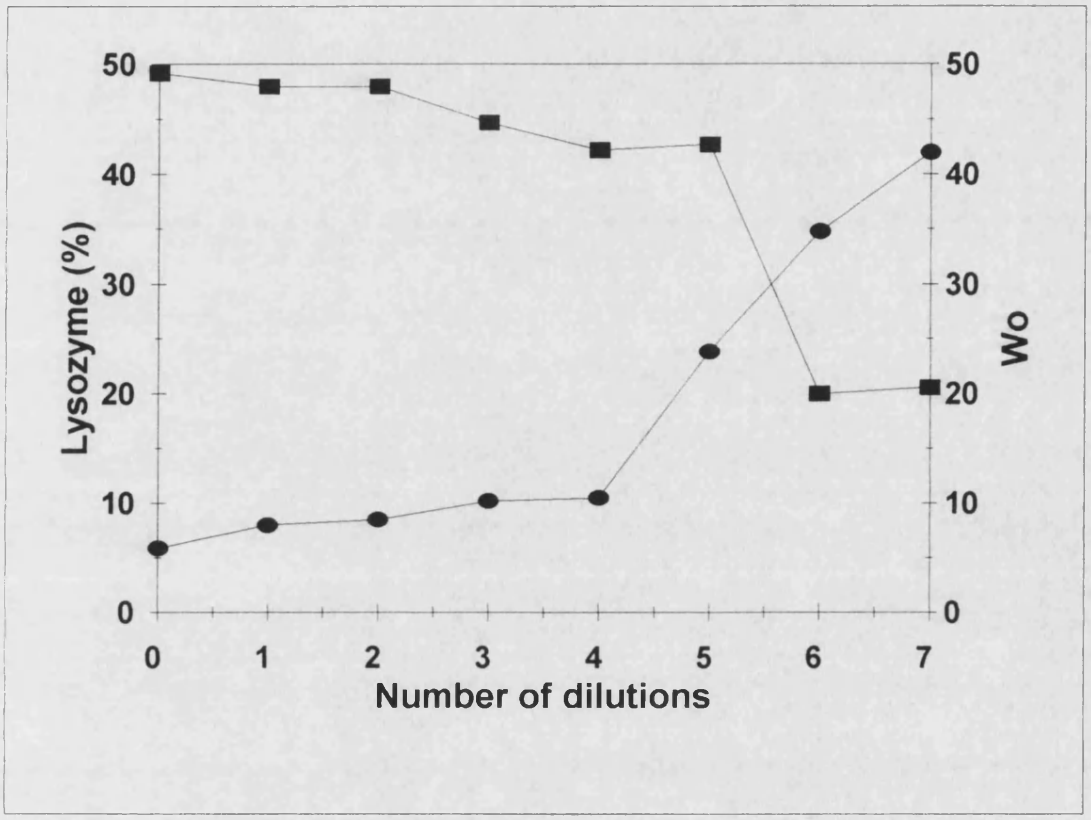

Figure 6.14. Dilutions inside 50 mM AOT/isooctane micelles after the forward transfer of reduced lysozyme in 2 M GuHCl

Removal of the denaturant from the reversed micelles involved a number of contacting stages (1-7) in which 5-10 ml of the reversed micelle phase containing the denatured and reduced protein was mixed with the same volume of an aqueous phase solution containing 0.1 M KCl or NaCl in 0.1 M Tris-HCl, pH 8.7. This step was repeated with the resulting organic phase and a fresh aqueous solution to remove any additional denaturant. After each contacting stage the protein content (■) and water content (●) were determined.

No further dilutions were performed in this experiment as after the 5th dilution, the protein present in the organic phase (40%) dropped to a value of 20% by the sixth dilution resulting in a precipitate at the interface. This is an important result since the number of dilutions can be optimised in order to prevent any loss in protein. The size of micelles formed with NaCl are larger than those formed with KCl, so subsequently dilutions performed using NaCl allow a greater dilution of the denaturant inside the micelle than KCl.

Experiments were also performed on the transfer of reduced and denatured lysozyme in 2 M GuHCl into 50 mM AOT/hexane reversed micelles. This was to determine whether the alkane phase could consist of another and show that one is not limited to the use of isooctane as the alkane phase. Figure 6.15 shows the dilution of denaturant inside a 50 mM AOT/hexane reversed micelle. After seven dilutions with reversed micelles containing 0.1 M KCl, the micelle size increased from a Wo value of 7 to 35. It was also observed that after each mixing time of 10 min the amount of protein in the organic phase remained constant at 90% and no protein was lost in the aqueous phase. However, after the 8th dilution the protein in the organic phase dropped to below 20%. Unfortunately, the concentration of denaturant inside the micelles was unknown but since the water content of the micelle had increased from a Wo value of 7 to 35 from the 1st to the 7th dilution, the denaturant would have been diluted by a factor of 5.

Hagen (1989) showed that GuHCl could be removed from reversed micelles by contacting the organic solution containing the denatured RNase with a fresh aqueous solution containing 0.1 M KCl. The advantage of these experiments was that GuHCl was labelled (^{14}C guanidine-hydrochloride) so it could be monitored inside the micelle. The results showed that 50% of the label could be removed after 4 contacting stages. In addition to removing the label the contacting stages with aqueous solution of low ionic strength increased the Wo from 4 to 9. The protein remained in the organic phase during this period. It was also found that if the number of contacting stages were extended from 4 to 10, a tenfold decrease in GuHCl concentration could be obtained.

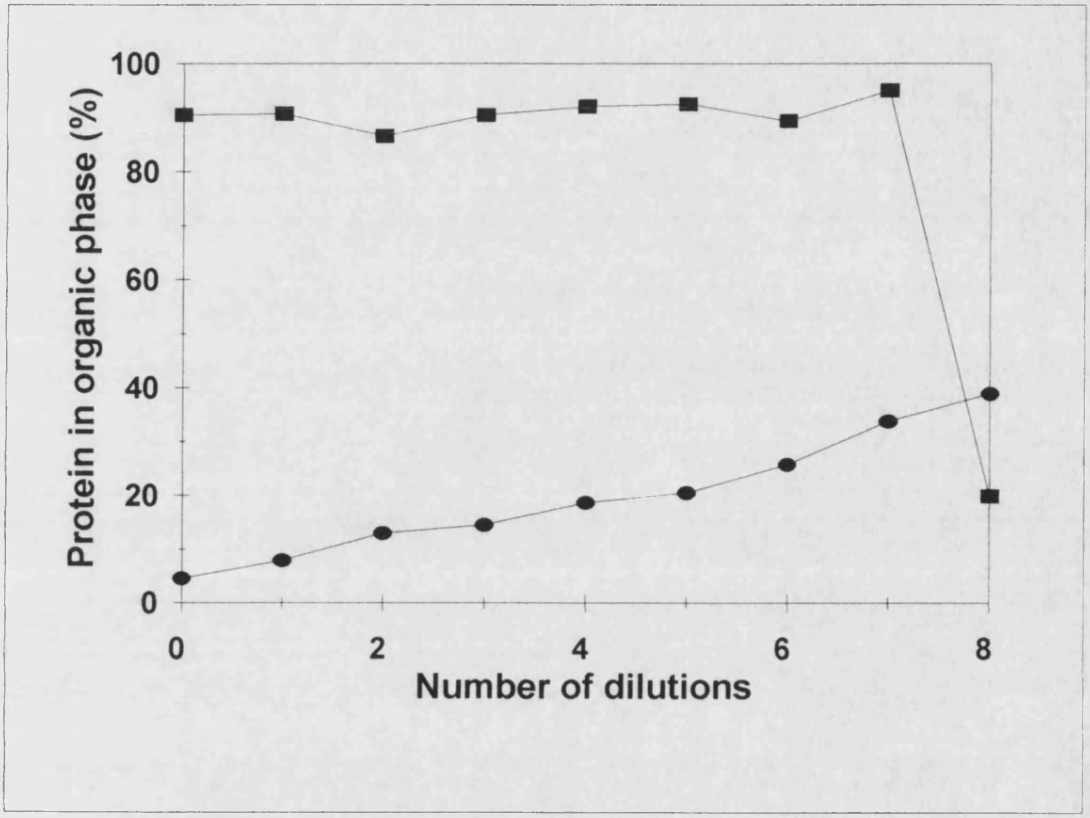

Figure 6.15. Dilution of denaturant inside 50 mM AOT/hexane reversed micelles

Removal of the denaturant was performed as in Figure 6.14. After each contacting stage, the protein content (■) and water content (●) were determined.

The results presented in this section did show an increase in micelle size and therefore water content indicating that dilution of the denturant was occurring but it was impossible to know the exact concentration of denaturant inside the micelle. The only way of determining the exact amount of transfer of GuHCl would have been to label the GuHCl (^{14} C guanidine-hydrochloride) or to use the direct injection transfer method for protein transfer into the micelles rather than the phase transfer method. The injection method has the advantage that the exact volume of the aqueous phase is known and that all the denaturant would be inside the aqueous core of the micelle as long as it did not have an affinity for the organic phase. Experiments were performed by injection of a concentrated solution of lysozyme denatured in GuHCl (1-6 M) into 50 mM AOT/isooctane but reversed micelles were not formed under the conditions employed here.

However, the micelle size was shown to increase with each contacting stage thus dilution of the denaturant was occurring inside the micelle. The number of contacting stages was also shown to be important as loss of protein from the organic phase occurs when the number is too high. A possible mechanism for the dilution of denaturant inside the micelle is the partial coalescence of the micelle with the surfactant layer at the interface between the organic and aqueous interface, Jada *et al.*, (1989). This allows small ions to exchange and at the same time allow water molecules to enter the micelle. The low ionic strengths used forces the protein to remain in the micelle as it departs from the interface.

6.4.2 The backward transfer of reduced lysozyme from reversed micelles

The backward transfer of reduced lysozyme using a 1 M KCl solution at a pH of 11.2 was also performed. Under these conditions it has been shown that native lysozyme can be successfully back transferred from a reversed micelle system regaining its full activity (Section 6.2). However, results obtained for the backward transfer of reduced protein show that between 0.2-1 M GuHCl 25% of the protein is recovered and between 1-3.2 M GuHCl there is a steady decline

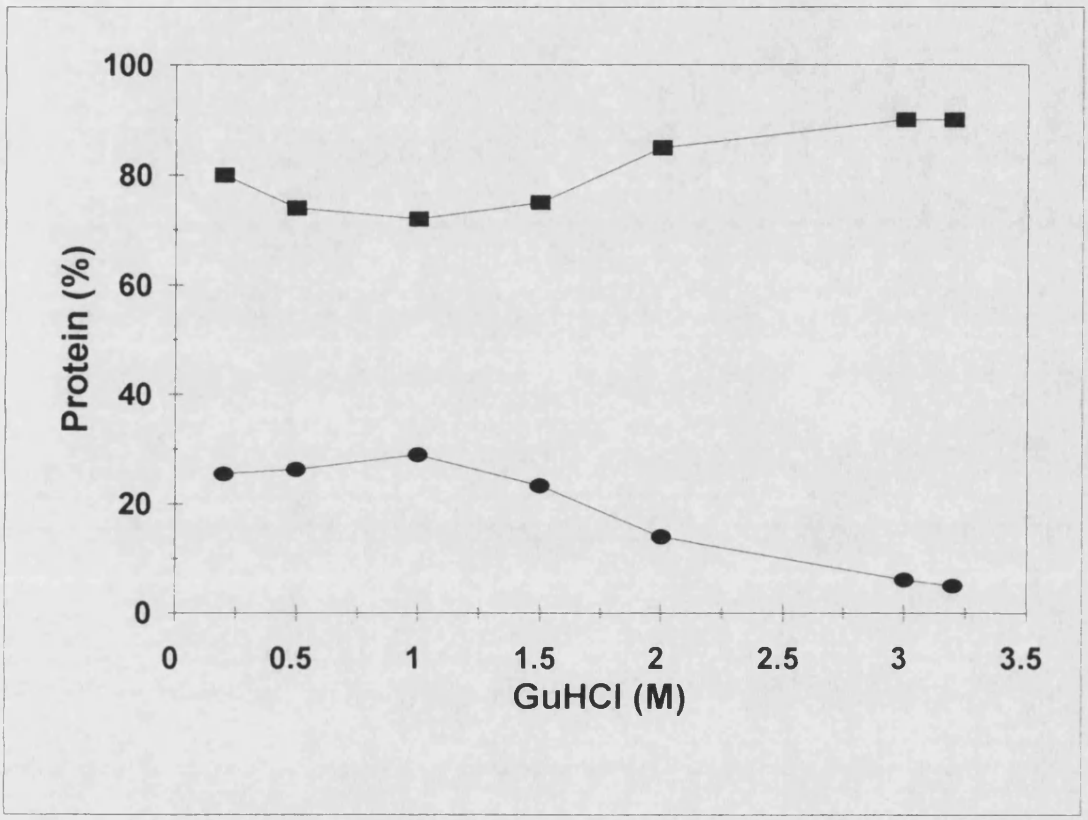

Figure 6.16. The backward transfer of reduced lysozyme inGuHCl from 50 mM AOT/isooctane micelles

The backward transfer involved mixing 4 ml of the organic phase containing the protein from the forward transfer with 4 ml of 1 M KCl in 0.1 M Tris-HCl, pH 11.2. The protein concentration was determined in the aqueous phase (●). The amount of precipitate formed at the interface was also quantified (■).

from 25-5% recoverd lysozyme (Figure 6.16). The protein concentration was measured in both phases after the backward transfer step and the organic phase was found to have very little or no protein present. Thus, most of the protein leaves the organic phase but very little transfers back into the aqueous phase. This protein in the aqueous phase had no activity. A solid white precipitate was observed at the interface which can be assumed to be 80-90% of the protein with some surfactant from a simple mass balance. This protein/surfactant complex was analysed using the two-phase titration (Section 4.2.1.1) and found to contain 2-5% of the surfactant. To transfer the reduced protein from the micelle into a fresh aqueous solution it requires the protein filled micelles to collide with the interface. Then, as the protein starts to leave the micelle the exposed hydrophobic parts of the protein must bind or interact with the surfactant tails preventing the recovery of the protein into the aqueous phase. It then forms a thick white precipitate at the interface.

The reduced form of lysozyme in these mixed denaturant systems has a more random coil conformation as the disulphide bonds are broken. When the back extraction was performed the surfactant was found to interact with the protein and form a precipitate at the interface. Once again the protein left the organic phase with 80% precipitating at the interface and 20% transferring back into the aqueous phase. No protein activity was detected for the lysozyme in the aqueous solution. These observations are consistent with the results presented for the backward transfer of reduced lysozyme in GuHCl.

Therefore, for the backward transfer of reduced lysozyme to be efficient the disulphide bonds need to be reformed so that the hydrophobic patches are hidden and the protein cannot interact with the surfactant and regain a native structure. It has previously been shown that non-reduced lysozyme can be successfully back transferred (Section 6.3.1). However, refolding the reduced form of the protein requires the formation of disulphide bonds in the correct maner and is more complicated.

To reform the native structure of lysozyme from the reduced state in reversed micelles the disulphide bonds require to be reformed inside the micellar aqueous core. For this to occur the denaturant concentration needs to be diluted to a minimum together with the correct oxidising conditions to permit the protein to return to a native environment. Once the protein was in the native form it could be back extracted into the aqueous phase without any interactions at the interface.

6.4.3 Disulphide bond formation

Once the denatured protein has been transferred into the micelles and the concentration of denaturant reduced the next stage is to reform the disulphide bonds enabling the protein to recover its native, active structure. Lowering the denaturant concentration provides the correct environment for the protein to reach a near native state. For disulphide containing proteins, such as lysozyme, reoxidation is necessary for the recovery of the three dimensional native structure. An important factor in thiol and disulphide bond chemistry is the pH. The majority of thiols have pK values of around 8 and 9 so they are most reactive at alkaline pH values and their reactivity decreases as the pH decreases.

Normally during *in vitro* folding the reagent used is glutathione which acts as the redox reagent. Oxidised glutathione (GSSG) is necessary for the formation of disulphide bonds and reduced glutathione (GSH) is required to break any incorrectly formed disulphide bonds. The presence of reduced and oxidixed glutathione allows reshuffling of the disulphide bonds to occur until the correct bonds are formed. The concentrations of the reduced and oxidised forms of glutathione can be varied depending on the protein studied but the concentrations required are generally 10 mM for the reduced form and 1 mM for the oxidised form (Saxena andWetlaufer, 1970).

The refolding of lysozyme in an aqueous solution has been investigated by a number of workers (Saxena and Wetlaufer, 1970, Goldberg *et al.*, 1994) and the conditions have been optimised for disulphide bond formation using 10 : 1 of the

reduced to the oxidised forms of glutathione. However, the optimum conditions required inside reversed micelles are unknown. Another problem in detecting disulphide bond formation inside the micelle is that there is no known assay that can be used. Activity measurements using *Micrococcus luteus* cannot be used with AOT/isooctane micelles since the solutions produced are yellow in colour and cannot form clear solutions on contact with the organic phase. This means there is no way of determining how active lysozyme is inside the micelle. The substrate used by Steinmann *et al.* (1986) for the measurement of activity of lysozyme inside AOT/isooctane reversed micelles was 3, 4-DNP-NAG. Unfortunately this substrate is no longer commercially available. CD was used to determine the structure of lysozyme in Chapter 4 and the extent of unfolding was found to be dependent on the denaturant used. Consequently, experiments were performed for refolding inside micelles followed by back extraction and measurement of activity in the resulting aqueous solution.

Table 6.1 shows the conditions that were employed in order to reform disulphide bonds inside the micelles. Under all the conditions tested the backward transfer resulted in the formation of a precipitate at the interface with no protein in the aqueous phase. After the backward transfer step it was noted that most of the protein (90-100%) had disappeared from the organic phase as measured by A_{280} and none was present in the aqueous phase. A thick precipitate was always observed in each sample at the interface. The precipitate consisted of the majority of the protein bound to some of the surfactant (2-5%) and this was proved by the two phase titration used in Chapter 4. This implies that the conditions employed did not enable the disulphide bonds to form inside the micelles and hence the protein remained in the unfolded form. Thus, on attempting to transfer the protein back from the organic phase the exposed hydrophobic patches on the protein molecule must have interacted wth the surfactant tails or head groups and formed the protein-surfactant complex. The results observed here are similar to those reported earlier on the backward transfer of reduced lysozyme from reversed micelles.

Table 6.1. Conditions of reduced and oxidised forms of glutathione used for forming the disulphide bonds in lysozyme inside rversed micelles

5 ml of the organic phase containing the reduced protein was mixed with 2.5 ml of reversed micelles containing glutathione prepared by injecting 50 µl of aqueous solution containing a range of mixtures of reduced and oxidised glutathione as shown into 1 ml of 50 mM AOT/isooctane. After this step the resulting solution was mixed with an equal volume of an aqueous solution containing 1 M KCl in Tris-HCl, pH 11.2 for 30 min for the backward transfer to be achieved.

Reduced glutathione (GSH)	**Oxidised glutathione (GSSG)**	**Backward transfer**	**Activity (%)**
1.5 mM	0.15 mM	precipitate at interface	0
1.5 mM	1.5 mM	precipitate at interface	0
3 mM	0.03 mM	precipitate at interface	0
3 mM	0.3 mM	precipitate at interface	0
3 mM	1 mM	precipitate at interface	0
3 mM	2 mM	precipitate at interface	0
3 mM	3 mM	precipitate at interface	0

Table 6.2. The effect of mixing time on the formation of disulphide bonds inside reversed micelles

Mixing times (24-120 hrs) were investigated on the extent of disulphide bond formation of lysozyme inside AOT/isooctane reversed micelles

Mixing time (hrs)	Backward transfer	Activity (%)
24	precipitate at interface	0
36	precipitate at interface	0
48	precipitate at interface	0
72	precipitate at interface	0
96	precipitate at interface	0
108	precipitate at interface	0
120	precipitate at interface	0

The above experiment was repeated using different mixing times (24-120 hrs) to establish whether the formation of the disulphide bonds required a longer period to reform (Table 6.2). However, on backward transfer of protein the result was always the formation of a precipitate at the interface. A temperature of 20°C was used in all the experiments and no other temperatures were investigted. It was shown in the previous section that refolding lysozyme in an aqueous environment was temperature dependant and the optimum recovered activity was achieved at 40°C. The effect of temperature was not studied in reversed micelles and perhaps an increase in temperature may have aided the recovery of active protein.

The pH of the environment in which refolding takes place is a major factor. The optimum pH of the aqueous solution for recovery of lysozyme activity is 8.7. However, inside the reversed micelle the pH is difficult to determine, therefore the experiment was repeated using a range of pH values from 7-10.5 (Table 6.3). Once again the backward transfer resulted in the formation of a precipitate at the interface.

Refolding experiments were also performed by changing the alkane phase from isooctane to hexane. The reason for this change is that by reducing the length of the oil, larger micelles are formed (Mall, 1993). An attempt was made to refold the lysozyme inside micelles composed of 50 mM AOT/hexane. The protein was reduced and denatured in 2 M GuHCl and transferred into the hexane micelles in the normal maner yielding between 90-100% of the protein in the organic phase. The denaturant was then diluted by a number of contacting stages as described in the Section 6.4.1. This resulting organic phase was then contacted with another organic phase containing the refolding buffer (reduced:oxidised glutathione 10:1). Several backward transfer experiments were performed (Table 6.4) at various time intervals (10 min-5 days) using both the ethyl acetate and high salt, high pH (1 M KCl at pH 11.2) methods for back extraction. In each experiment the protein was found to leave the organic phase, as verified by A_{280} measurements but none was recovered in the aqueous phase. In all cases a thick precipitate was formed at the interface as occurred previously for the AOT/isooctane micelles. By changing the

Table 6.3. The effect of pH on the formation of disulphide bonds in lysozyme in AOT/hexane reversed micelles followed by backward transfer

A number of pH values (7-10.5) were investigated for disulphide bond formation followed by backward transfer. The pH (wp) is the calculated pH inside the water pools of the micelles using Equation 2.12.

pH (buffer)	pH (wp)	Backward transfer	Activity (%)
7.0	6.94	precipitate at interface	0
7.5	7.27	precipitate at interface	0
8.0	7.6	precipitate at interface	0
8.5	7.93	precipitate at interface	0
9.0	8.26	precipitate at interface	0
9.5	8.59	precipitate at interface	0
10.0	8.92	precipitate at interface	0

Table 6.4. The effect of mixing time on the formation of disulphide bonds in lysozyme in AOT/hexane reversed micelles followed by backward transfer at different time intervals

Refolding experiments were performed on lysozyme inside 50 mM AOT/hexane reversed micelles by contacting with an organic phase containing refolding buffer (reduced:oxidised glutathione 10:1). The backward transfer experiments were then performed at different time intervals (10 min-5 days).

Time (hrs)	Backward transfer	Activity (%)
0.16	precipitate at interface	0
12	precipitate at interface	0
24	precipitate at interface	0
36	precipitate at interface	0
48	precipitate at interface	0
96	precipitate at interface	0
120	precipitate at interface	0

alkane phase from isooctane to hexane no improvement in the refolding behaviour of lysozyme was found.

It must be noted that in the results presented so far the denaturant and reducing agent were removed by dilution. The protein did not refold under any of the conditions presented. Hagen (1989) has shown that the activity of RNase may be recovered upon addition of glutathione in reversed micelles. The advantage of this work was that activity could be measured inside the micelles and this reached a maximum after 20-30 hours. The optimum ratio of GSH/GSSG was 3:1 which gave 100% activity for RNase.

Refolded lysozyme could not be recovered in the aqueous phase. The number of contacting stages required for dilution prior to reformation of the disulphide bonds were also limited to 8 since protein was lost from the organic phase after this stage. Hagen (1989) showed that after 7 contacting stages there was a decrease in the ability for the protein to refold. This could have been attributed to the effect of prolonged shear and mixing since the volume of sample was reduced as a result of loss at the interface associated with phase separation especially towards the end of the experiment. This means that the number of contacting stages needs to be optimised in order to avoid detrimental shear effects.

6.4.4 Refolding in the presence of AOT

The effect of AOT concentration on the activity of lysozyme was investigated (Figure 6.17). The activity of native lysozyme falls to between 10-25% as the concentration of AOT is raised to 2 mM. This is a significant result and shows the detrimental effect of AOT on activity. Even though the protein is in its native state, AOT concentrations exceeding 2 mM must affect the sructure of the protein. This could arise by firstly ionic interations between the ionic surfactant headgroup and hydrophillic parts on the surface of the protein. Secondly, as the surfactant concentration increases it must penetrate the interior of the protein. The hydrophobic tail of the surfactant may now interact with hydrophobic parts of the

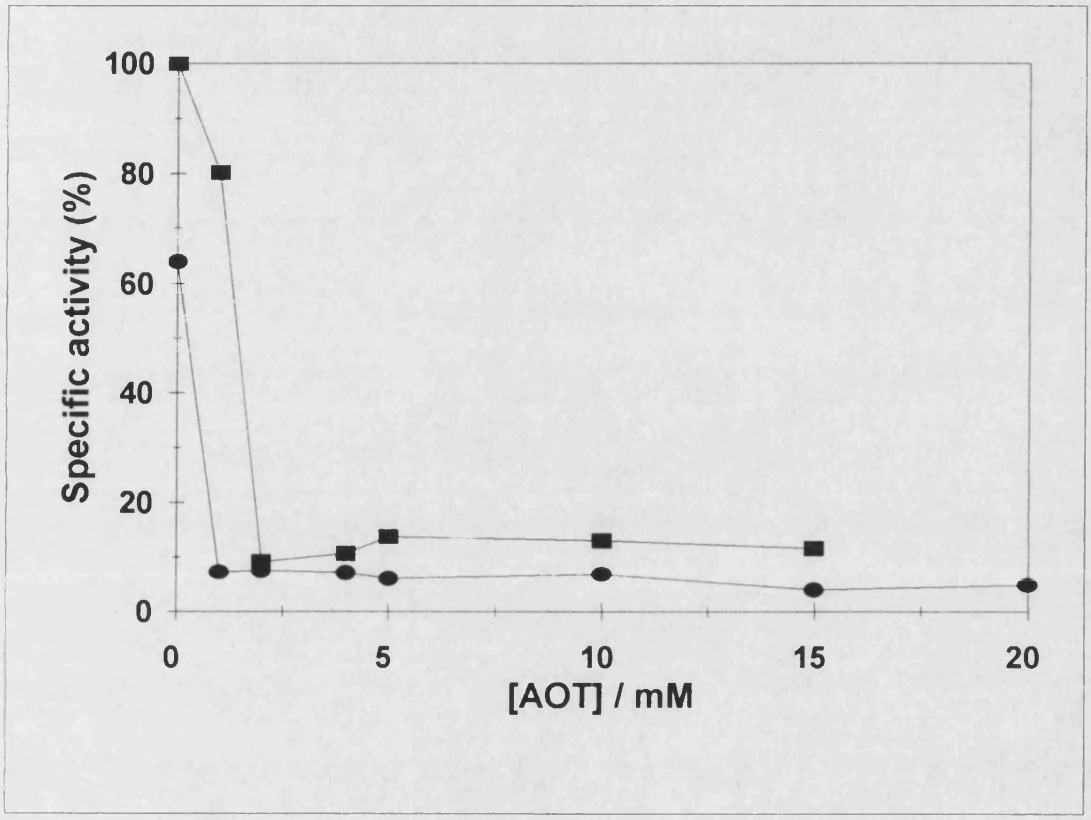

Figure 6.17. The effect of AOTconcentration on the refolding of lysozyme

Lysozyme (■) 0.015 mg/ml was prepared in 25 mM phosphate buffer pH 7.0 with increasing concentrations of AOT (1-20 mM). Lysozyme was also denatured and reduced (●) in 6 M GuHCl in the presence of increasing concentrations of AOT (1-25 mM). Enzyme activity was assayed and expressed relative to native lysozyme at the same concentration.

molecule causing further unfolding and denaturation. Thus, the surfactant concentration must be reduced to below 2 mM (preferably zero) for the protein to retain its activity.

Lysozyme denatured and reduced in 6 M GuHCl in the presence of AOT cannot be reactivated in AOT concentrations exceeding 1 mM (Figure 6.17). In the absence of surfactant, lysozyme refolds and recovers 65% of its activity. Between AOT concentrations 1-20 mM the recovery of lysozyme activity only reaches values of <10%. As lysozyme is in its fully reduced form and is completely unfolded in 6 M GuHCl, its structure can be assumed to be randomly coiled. This allows the hydrophobic tail of the AOT to interact and bind to the hydrophobic parts of the unfolded molecule. The ionic head groups must also bind to the hydrophillic parts of the protein. The unfolded protein molecule allows AOT to interact at much lower concentrations (1 mM) than with the native protein (2 mM) and prevents recovery of enzyme activity. A possible explanation for these results is that the negatively charged AOT head groups attach themselves to the hydrophillic parts on the protein via ionic interactions. Further increases in AOT cause more binding and hence more unfolding causing gross changes at the active site and gross conformational changes.

Tanford (1980) has proposed three modes of interaction between proteins and detergents. These are described below:

1. Surfactants can associate with specific binding sites on the native protein. These inteactions can be electrostatic and/or hydrophobic.
2. There can be a cooperative association between the protein and a large number of denaturant molecules without any major conformational changes occurring to the protein. This type of interaction has been observed between serum albumin and alkyl sulfates and sulfonates with short hydrocarbon chains (C_8, C_{10}).
3. There can be a cooperative association between the protein and detergent molecules causing denaturation of the protein. The native structure of the protein is destroyed and replaced by a rodlike structure with high α-helix content. The hydrophobic residues of the protein are able to associate with the detergent

molecules. This type of interaction has been observed with SDS and several proteins and is described later.

Treves *et al.* (1983) have studied the effect of a number of surfactants on the activity of several proteins. They found that the long chain anionic detergent SDS was a strong inhibitor of lactase dehydrogenase, malate dehydrogenase and isocitrate dehydrogenase. The surfactants were used at a concentration of 2.5 mM in these studies. The two cationic surfactants dodecyl-trimethylammonium bromide and 2, N-benzyl-N, N-dimethyl-N-(4-(1,1,3,3-tetramethylbutyl)-phenoxyethoxyethyl) ammonium chloride only inhibited lipase extensively by 50%. Treves *et al.* (1983) concluded from their work that the interactions between detergents and enzymes are based on chemically selective reactions which are influenced by the structure of the protein and the detergent. How the detergents interact with enzymes depends on the concentration of detergent employed.

Creagh *et al.* (1992) have investigated the effect of aqueous surfactant solutions on alcohol dehydrogenase (LADH). They showed from fluorescence data that LADH in aqueous solutions of sulfate and sulfonate surfactants with short hydrocarbon chains had little effect on the conformation of the protein. However, when long- and branched-hydrocarbon chain surfactants were used a decrease in the fluorescence was observed. This was conclusive evidence that there were conformational changes and denaturation of the protein occurring in these aqueous surfactant solutions, especially AOT and SDS. CD studies also confirmed that the short chain surfactants have little or no effect on the protein, whereas the long- and branched-hydrocarbons show denaturing effects.

There are several mechanisms by which the surfactant could interact with lysozyme. As mentioned earlier adsorption of the surfactant could take place through electrostatic and/or hydrophobic interactions with or without denaturation. AOT may interact with lysozyme via two mechanisms. The AOT probably adsorbs to the surface of the protein followed by some active site alterations.

Thus, for refolding proteins inside reversed micelles, the protein must refold to its tertiary structure inside the micelle leaving no hydrophobic patches exposed that can interact with the hydrophobic tails of the surfactant. This will reduce the number of binding sites available for AOT to interact with inside the micelle. It is also important after back extraction of the native protein that the AOT concentrations must be less than 2 mM in order for activity to be regained. Preferably there should be no surfactant present in the fresh aqueous solution. Lysozyme has been shown to retain all of its activity after backward transfer from AOT reversed micelles (Section 6.2.1.1) thus it can be assumed that there must be less than 1% AOT in the aqueous solution and the majority of the surfactant must be redistributed at the interface.

6.4.5 Summary

Lysozyme in the non-reduced form regained full activity after the backward transfer into an aqueous solution using GuHCl and mixtures of GuHCl and urea. The presence of the disulphide bonds restricted the protein from having a more randomly coiled structure. The majority of protein was successfully recovered and 100% activity was regained due to a 100 fold dilution going from the small aqueous core of the micelle to the aqueous phase. This dilution simply washed away the bound denaturants from the protein and allowed it to return to its native environment. These results indicate that there is potential for refolding non-disulphide containing proteins inside reversed micelles. As long as the protein is partitioned in the fully unfolded form and micelles can be used to prevent the aggregation that occurs in an aqueous environment this method may be extended to other proteins.

An attempt has been made to refold denatured and reduced lysozyme inside AOT/isooctane reversed micelles. Denatured and reduced lysozyme has been successfully partitioned into these systems using a variety of denaturants. Once the protein was inside the micelles dilution with the appropriate buffer was monitored by Karl-Fischer titration. After each dilution the micelle size was observed to

increase due to the increase in water content. This resulted in the denaturant and DTT concentations being diluted as a consequence of increased water content. However, it was difficult to know exactly how much denaturant was present inside the micelle as no appropriate method was available. The only certain fact was that the dilution factor inside the micelle which ranged between 7-10. An important parameter was the number of contacting stages that were required for the dilution. This was optimized, since too many contacting stages caused a loss of protein from the organic phase resulting in a precipitate being formed at the interface.

After the required number of dilutions an attempt was made to reform the disulphide bonds inside the micelle. There was no assay available to monitor reoxidation of the disulphide bonds under these conditions. A number of different ratios of the reduced and oxidized forms of glutathione were used at different concentrations to form reversed micelles. The glutathione containing reversed micelles were mixed with the protein containing micelles which should have allowed the micelles to mix the contents together due to their dynamic nature. However, after backward extraction of the protein, a precipitate was formed at the interface which was shown to be some surfactant and most of the protein. Thus the protein was still thought to be in the unfolded form and the exposed hydrophobic patches were able to interact with the surfactant interface, forming a protein/surfactant complex. There are two possible reasons for this occurring. Firstly, the number of dilutions required to remove the denaturant from the micelles were not enough, and the protein still had denaturant bound to it. Secondly, the conditions used for disulphide bond formation inside the micelles are not the same as for an aqueous solution. Parameters such as pH and mixing time for disulphide bond formation resulted in no recovery of the protein and a protein/surfactant complex formed at the interface.

Finally refolding lysozyme in the presence of AOT was investigated. Native lysozyme in the presence of AOT below 2 mM retained all of its activity. However, concentrations exceeding this showed the detrimental effect the surfactant has on the protein. Refolding lysozyme in the fully unfolded state in the

presence of AOT showed that a lower concentration of AOT (<1 mM) resulted in a major loss of activity. This clearly illustrates the requirement for complete removal of surfactant if micelles are going to be used for refolding.

Reduced and denatured lysozyme could not be refolded under the conditions employed here. One of the problems associated with this work was that no substrate was available to monitor lysozyme activity inside the micelles. Consequently, activity had to be measured after recovery of the protein from the micelles. Since no protein could be recovered after dilution and reoxidation of the disulphide bonds the folding problem must be associated with one or both of these steps.

CHAPTER 7
CONCLUSIONS AND FUTURE WORK

In this study reversed micelles were used in an attempt to isolate protein molecules from each other during refolding and hence reduce the intermolecular interactions causing aggregation. Lysozyme was chosen as the model protein due to the wealth of literature available regarding its refolding behaviour and its ability to partition into reversed micelles. The first step was to establish the conditions for the transfer of native and non-native lysozyme (non-reduced and reduced). The next step involved removal of the denaturant inside the micelles by a number of the contacting stages of the organic phase with a fresh aqueous phase. Attempts were then made to provide the correct redox conditions to allow the disulphide bonds to form. Finally, the protein was recovered by contacting the organic phase with an appropriate aqueous solution.

The results have already been summarised in the appropriate chapters hence only the important conclusions will be drawn in this chapter. Also recommendations for future work in this field will be included.

7.1 Partitioning, characterisation and backward transfer of native lysozyme

The transfer of native lysozyme showed pH, contact time, ionic strength and surfactant concentration to be the crucial parameters governing the transfer into reversed micelles composed of AOT/isooctane. It is believed that electrostatic interactions between the protein and inner layer of the micelle control this solubilisation process. Native lysozyme (90%) was successfully transferred into AOT/isooctane reversed micelles and recovered back into a fresh aqueous solution regaining its tertiary structure as monitored by CD and 100% enzyme activity. These findings were important because once the protein was refolded inside the micelle, recovering the protein could be simply made by adjusting the pH or using ethyl acetate mediated transfer.

7.2 Partitioning and refolding of non-reduced lysozyme

The transfer of lysozyme in the non-native form with its disulphide bonds intact transferred into reversed micelles in a similar fashion to the native form of the protein. The disulphide bonds restrict the protein from having a more random coil structure and contribute to its stability. The major problem using this transfer step was that the electrostatic interactions between the positively charged guanidinium ion and the negatively charged AOT surfactant headgroups caused the micelles to shrink and size-exclude the protein. Lysozyme was transferred into these micelles up to a concentration of 1 M GuHCl. CD and fluorescence data showed that no major structural changes occurred upto 3 M GuHCl. However, changes did occur between 3-6 M GuHCl which were complete at 6 M GuHCl. This meant that lysozyme needed to be partitioned into the micelles at 6 M GuHCl to be completely unfolded. Structural data showed lysozyme was transferred in the partially unfolded form into the micelles at concentrations of 1 M GuHCl. Alternative methods had to be developed in order to partition lysozyme in the completely unfolded state. These are discussed below.

7.2.1 Mixed micelles

This section draws the major conclusions from Chapter 5 using novel mixed surfactant systems in order to improve the transfer efficiency of denatured lysozyme into reversed micelles. Mixed surfactant micelles were formed using mixtures of AOT with Span 85, Tween 85 and Tween 20. However, the Span 85 system was discarded since the transfer of lysozyme dropped from 90% to 40% by increasing the Span 85 content from 1-2% in the mixture (AOT:Span85). The AOT+Tween 85 and AOT+Tween 20 systems proved to be less efficient at transferring lysozyme in GuHCl than the AOT system alone. The size of these mixed micelle systems showed that increases in the nonionic counterpart in the mixture resulted in poorer transfer due to the decreased size of the water-pools in

the micelles. These mixed micelles were found to be smaller in size than the AOT micelles alone.

7.2.2 Mixed denaturants

This section draws the major conclusions from the work in Chapter 5 with regards to improving the transfer of denatured lysozyme using a mixed denaturant system consisting of GuHCl and urea at different ratios and concentrations. The transfer of non-reduced lysozyme in a number of mixtures GuHCl:urea occurred at higher denaturant concentrations than could be used with GuHCl alone. By reducing the protein, there were further increases in the mixed denaturant concentration at which transfer occurred. This occurred due to the opening of the molecule into a more randomly coiled configuration which allowed additional binding sites for the denaturant. The size of the micelles formed with the mixed denaturant systems studied showed that the size of the water-pool decreased as the GuHCl content increased in the mixture of GuHCl:urea. Fluorescence studies showed that the unfolding power of mixtures of GuHCl:urea lie between urea and GuHCl individually. They are more effective at unfolding lysozyme in urea but not as effective as GuHCl.

This novel system using mixtures of GuHCl and urea allowed the partitioning of lysozyme into AOT/isooctane micelles at higher denaturant concentrations than was previously allowed. Structural data of lysozyme showed that all the tertiary structure was lost in the reduced form in a number of these mixed denaturants. Lysozyme could now be partitioned into the micelles at high denaturant concentrations which solved the initial step before refolding inside micelles.

7.2.3 Refolding

Lysozyme in the non-reduced state was refolded completely from both GuHCl and urea by a 100 fold dilution. Lysozyme (non-reduced) in mixtures of GuHCl and urea recoverd full activity on recovery of the protein from the reversed micelles.

This involved a 100 fold dilution on backward transfer from the micellar phase to the aqueous phase. This shows that there is potential for refolding non-disulphide containing proteins. The proteins could be partitioned into the micelles at high mixed denaturant concentrations after which recovery in an aqueous phase would result in the native conformation of the protein.

7.3 Partitioning and refolding of reduced lysozyme

The transfer of non-native lysozyme with its disulphide bonds reduced behaves completely differently to the native and non-native (non-reduced) forms of the protein. The breaking of the bonds allows the protein to adopt a more random coil conformation. Electrostatic interactions did not govern this solubilisation process as for the native form of the protein. The reduced form of lysozyme transferred into the micelles at acidic pH (1.5-2.2). At these acidic values far from the pI of lysozyme (10.9), the protein possesses alot of negative charges. Since the protein is reduced the molecule is unfolded and covered with these negative charges. This creates more binding sites for the positively charged guanidinium ions. Lysozyme in GuHCl partitioned into the micelles upto 3 M GuHCl. This concentration was still not high enough for complete unfolding as monitored by CD and fluorescence. Urea was found to be incompatible with the phase transfer of lysozyme. Alternative methods were developed to overcome this problem and these are shown in the next section.

Lysozyme in the reduced form only refolds (in an aqueous environment) to an optimum (65%) after 1 hr at 40°C. Increases in temperature showed no further increases in activity. This optimum recovery (65%) was achieved at 0.015 mg/ml lysozyme and increasing the protein concentration for refolding drastically decreased the reactivity of the recovered protein. The reduced form of lysozyme could not be completely refolded even at the lowest protein concentration (0.015 mg/ml). This was due to the competing aggregation side reaction. It is for this reason that reversed micelles were chosen in an attempt to reduce this problem and refold at higher protein concentrations than can be achieved at present.

After the transfer of the reduced lysozyme into the micelles dilution of the denaturant were followed by Karl-Fischer titration. The water content increased 7 fold after 6-7 contacting stages with the dilution buffer. However, too many contacting stages resulted in loss of protein from the organic phase. After these dilutions attempts were made to recover the protein from the micelles using high pH and ethyl acetate. In each case a precipitate was formed at the interface containing 80-90% of the protein together with 2-5% of the surfactant. No active protein was recovered in the aqueous phase. This protein could not be recovered after the dilution process since the disulphide bonds were still reduced. These would have to be formed before recovering the lysozyme. Parameters such as mixing time, alkane phase and pH did not reform these bonds inside the micelles and recovery always resulted in the protein/surfactant complex. The protein did not reform the disulphide bonds under any of the conditions employed and attempts to recover the protein resulted in the protein/surfactant complex. Since the protein did not refold inside the micelle, it was concluded that the exposed hydrophobic groups inside the micelle could bind to the hydrophobic tails of the surfactant and form this irreversible protein/surfactant complex.

From the literature AOT is the most commonly used surfactant for the formation of reversed micelles. This is probably a poor choice since native lysozyme in an aqueous solution in the presence of AOT (> 2 mM) looses the majority of its activity. Denatured and reduced lysozyme cannot be refolded to regain its 65% activity even when 1 mM AOT was present. It appears that the disulphide bonds did not reform and on attempts to recover the protein, the unfolded form of lysozyme interacted with the AOT and formed the protein/surfactant complex. Unfortunately no assay was available to measure disulphide bond formation inside the micelles. The presence of AOT on the refolding of lysozyme showed to have a detrimental effect on activity. This means that if micelles are going to be used for refolding proteins, the surfactant would need to be completely removed in order for the protein to regain activity.

7.4 Future work

- Non-reduced lysozyme was found to regain full activity after backward transfer from the micelles into a fresh aqueous solution. There seems to be potential for refolding non-disulphide containig proteins such as carbonic anhydrase inside these aqueous droplets. Non-disulphide proteins could be partitioned in the unfolded form into micelles which would solve the problem of aggregation. A back extraction would recover the protein in its native state by diluting the denaturant concentration 100-fold.

- A major problem identified in this study was in monitoring the state of the protein inside the reversed micelle to carry out further fundamental studies. An assay needs to be developed in order to monitor disulphide bond formation and activity of lysozyme inside micelles. Ideally, the proteins studied in future should have an activity that can be assayed inside reversed micelles in order that refolding may be studied in detail.

- It was not easy to measure how much of the denaturant actually partitioned into the micelles. GuHCl binds to the unfolded parts of lysozyme and partitions into the micelles. In theory even if only 10% of a 1 M GuHCl solution is transferred into the micelles with the lysozyme, the actual concentration of denaturant inside the micelles will be 10 M based on the aqueous core volume containing 1% water. This problem could be solved by using a ^{14}C-guanidine hydrochloride label to monitor the transfer of denaturant into the micelles.

- A problem with using AOT as the surfactant is that it posseses negatively charged head-groups. Once the protein is in the unfolded form, there exists the possibility for binding of these groups onto the lysozyme. If non-ionic micelles were used for this process where the surfactant head-groups possessed no charges there may be fewer chances for binding of the protein and surfactant to occur. This could solve the problem of forming the protein/surfactant complex on recovering the protein. An option is to use naturally occurring surfactants

(phosphatridyl choline) should be considered when using reversed micelles for refolding.

- The transfer of lysozyme in GuHCl into AOT/isooctane reversed micelles was limited by the charge between the guanidinium ion and the AOT head group. This caused the micelle to shrink and expel the protein. To overcome this problem alternative denaturants for unfolding proteins that possess no charge need to be investigated. These denaturants need to be also compatible with the reversed micellar system chosen. Appendix 3 reports results on another novel system using DMSO as a denaturant. Mixtures of DMSO and water partitioned lysozyme into AOT/isooctane reversed micelles. Again structural data using CD and fluorescence regarding the conformation would need to be investigated.

- It is the interactions between the surfactant and protein that seem to be very specific and it therefore becomes important to carry out an analysis using a wide range of surfactants that have different characteristics. This will enable one to gain more information on the specific interactions that occur between proteins and surfactants and interferences caused during refolding. Once this information has been gained, rules can be developed to predict types of proteins that could be used with different reversed micelles (Appendix 2) to reduce the aggregation problem and increase refolding in biotechnology.

- Once a working system is established, the engineering aspects for the whole process would have to be investigated. These would include the quantification of the rates of forward and backward transfer of the protein in and out of micelles and refolding rates for the protein. Also any rate limiting steps in the process would need to be established. After these considerations, the design and operation on a larger scale could to be handled and again yields, rates, the recycling of the solvent and surfactant would have to be considered in order for the whole process to be efficient.

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APPENDIX 1

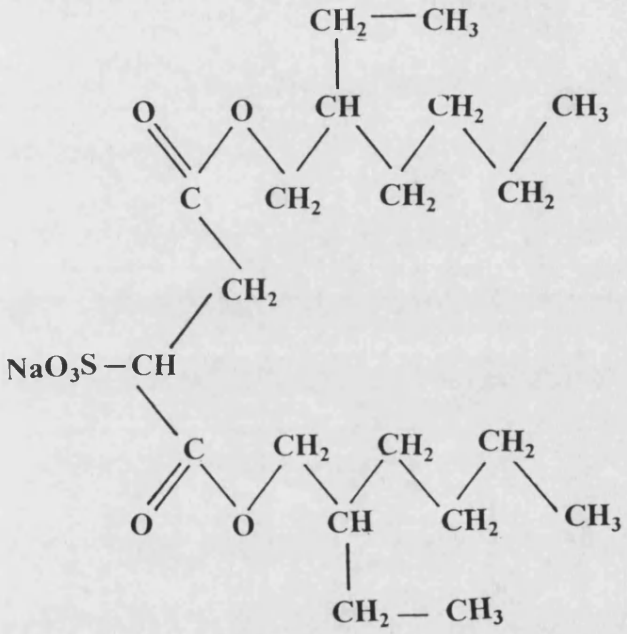


Figure A1.1 Structure of AOT

APPENDIX 2

The transfer of lysozyme into reversed micelles formed with other alkanes

AOT was found to form reversed micelles in other alkanes as well as isooctane. An optically clear, thermodynamically stable solution was formed when transferring lysozyme from an aqueous phase into a reversed micellar phase using the phase transfer method. Three alkanes including methylpentane, hexane and dodecane were used for the transfer of lysozyme (1 mg/ml) into 50 mM AOT/alkane solutions. Figure A2.1 shows the transfer of lysozyme into each of the reversed micelle systems as a function of salt (KCl) concentration. For the methylpentane and hexane reversed micelles, 80-90% of the protein was transferred between 0.1-0.4 M KCl and there is a large decrease in transfer to 30% at 0.6 M. At 1 M KCl, no protein is transferred into the hexane reversed micelles. The reversed micelles formed with dodecane allow 80% of the protein to be transferred at concentrations 0.6 M KCl then there was a steady decline in transfer as the KCl concentration was increased. The decrease in the transfer of lysozyme into micelles at higher KCl concentrations can be related to the electrostatic interactions between the AOT head groups and the charge on the salt used. Figure A2.2 shows the size of the reversed micelles formed after transfer of lysozyme. At 0.1 M KCl the dodecane reversed micelles are the largest with a Wo value of 21 then hexane with a Wo of 17 and methylpentane are the smallest with a Wo value of 16. However, as the KCl concentration was increased the size of the micelles formed are found to decrease for all three alkanes and these are all similar in size between 0.4-1 M KCl. The electrostatic interactions between KCl and the surfactant head groups reduces the repulsion between individual head groups and the micelle shrinks in size resulting in a decrease in protein transfer. The current literature lacks the research into the different types of reversed micelles that may be formed by changing the oils, surfactants and proteins used. The data shows that protein transfer is not limited to the widely used AOT/isooctane system.

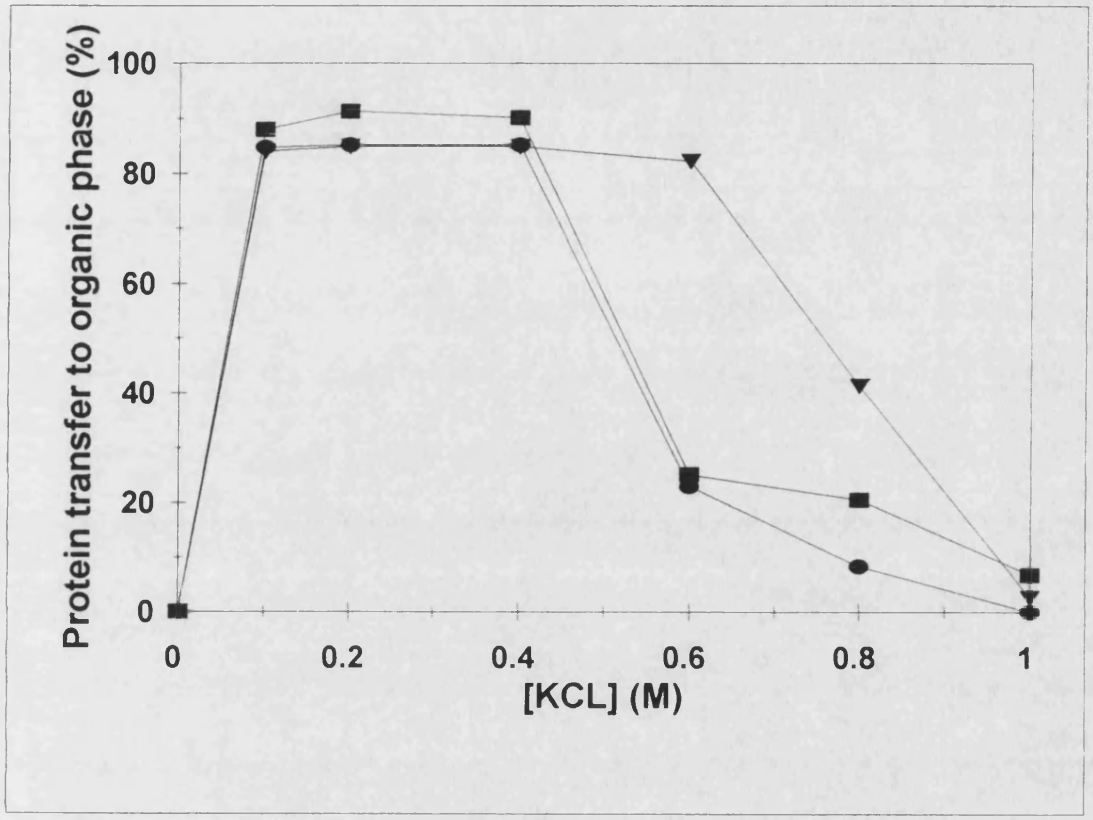


Figure A2.1. The effect of various alkanes on the forward transfer of lysozyme

Forward extraction experiments were performed by mixing equal volumes (5 ml) of 50 mM AOT/alkane (the alkanes were ■ = methylpentane, ● = hexane and ▼ = dodecane) with an aqueous phase (1 mg/ml protein in 25 mM phosphate buffer, pH 7) containing different KCl concentrations (0.1-1 M). The mixing time was 15 minutes. The results are an average of two determinations.

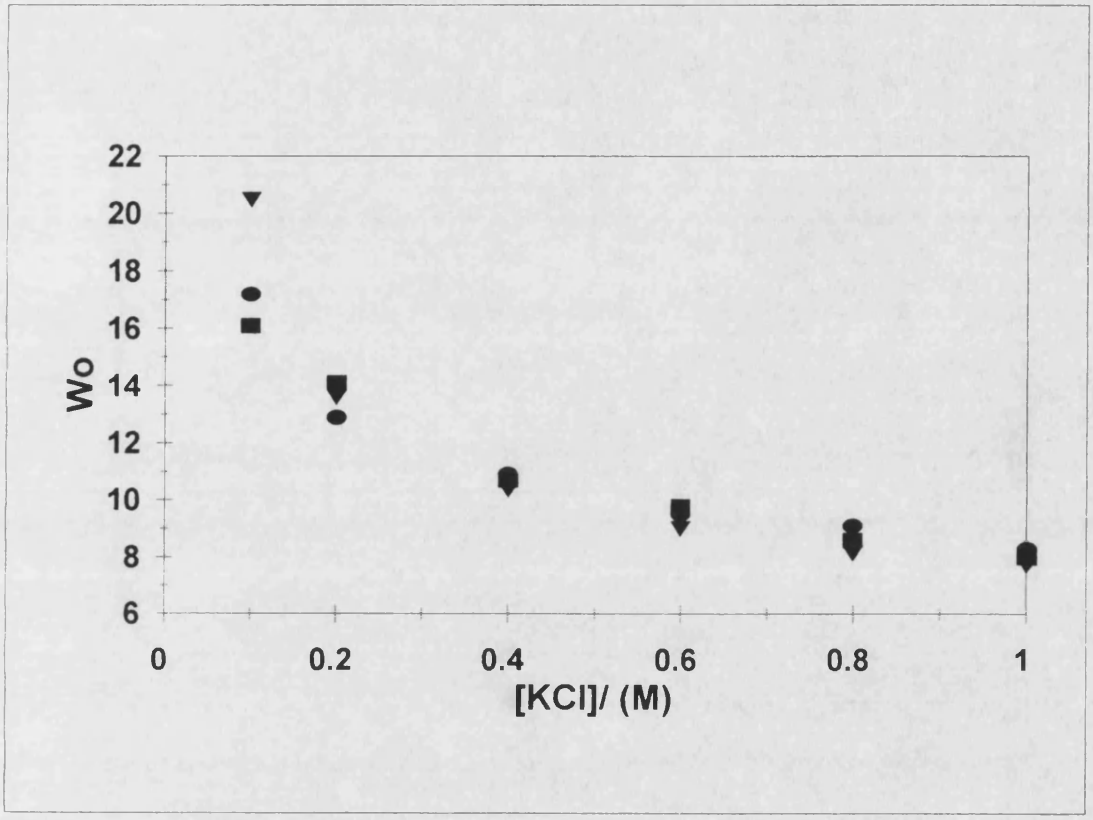


Figure A2.2. The effect of various alkanes on the size of the reversed micelles formed

Measurement of the water content inside the micelle was studied by mixing equal volumes (10 ml) of 50 mM AOT/alkane (the alkanes were ■ = methylpentane, ● = hexane and ▼ = dodecane) with KCl (0.1-1 M) in 25 mM phosphate buffer, pH 7.0. The samples were mixed for 12 hr to give constant values for water and cations in the organic phase then 0.05 ml of the organic phase was injected into the Karl-Fisher which calculated the amount of water present inside the micelles.

APPENDIX 3

Alternative denaturants for transferring lysozyme into micelles

This section presents results on two other possibilities for transferring denatured protein into reversed micelles. The first utilises mixtures of DMSO and water, whilst the second uses GuHCN which is supposedly more powerfull than GuHCl.

DMSO

The presence of urea prevented formation of reversed micelles using the phase transfer method. An alternative possibility was to use dimethyl sulfoxide (DMSO) as a denaturant. Recently Kotik et al., 1995 have compared the refolding of hen lysozyme from DMSO and GuHCl. DMSO unfolding of lysozyme was found to be similar to that obtained using GuHCl. The transfer of lysozyme using various ratios of DMSO:Water was performed (Table A3.1).

A complete DMSO system gave no transfer of lysozyme into the micelles (result not shown in table). An attempt was made to increase the KCl concentration to values between 0.1-1 M but it was found that still no protein partitioned into the micelles. However, by increasing the water content in the mixture (DMSO:Water) to a 5:5 ratio 98% transfer was achieved. At a ratio of 7:3 (DMSO:Water) very little protein was found in both the organic and aqueous phases and a large interfacial precipitate was visualised after the phases separated. This result indicates that the majority of the protein interacts with the surfactant at the interface to form this precipitate. There appears to be an optimum DMSO:Water ratio (5:5) which gives an excellent transfer of protein (> 90%) and increasing the DMSO above this content drastically reduces protein transfer.

The extent of denaturation in these conditions needs to be evaluated to know the exact affect of the DMSO:Water systems. Activity measurements together with structural analysis techniques such as fluorescence and circular dichroism would

show the effects of DMSO:Water on lysozyme. No further experiments were carried out using these systems but there seems to be potential in exploiting this area of research.

GuHCN

An alternative denaturant that can be used to unfold lysozyme is GuHCN. This is related to GuHCl in its structure but acts at a significantly lower concentration than GuHCl for some proteins, Pace (1975). The presence of salts have different effects on proteins depending on their type and concentration. At low ionic strengths, electrostatic interactions can stabilise proteins whereas at higher ionic strengths specific effects may be seen resulting in either salting in (solubilisation) or salting out (precipitation or crystallisation) Arakawa and Timasheff, (1984a). The addition of salts can cause an increase in hydrophobic interactions by what is known as the Hofmeister series. Destabilisation or salting in effects are normally caused by the extensive binding of the salt to the protein causing it to unfold. GuHCl has two effects when unfolding a protein. Firstly, it increases the solubility of the hydrophobic side chains which are normally present in the interior of the protein. Secondly, the hydrogens on the guanidinium ion compete with water for hydrogen bonding to the peptide bonds on the protein. As the salt concentration increases additional binding sites are made available and unfolding becomes a cooperative process.

A forward transfer experiment was performed to evaluate whether an increased amount of protein could be transferred into micelles at higher concentrations of GuHCN. The transfer of lysozyme into 400 mM AOT/isooctane micelles using the denaturants GuHCl and GuHCN is shown in Figure A3.2. At a concentration of 2 M GuHCl 78% of the protein was transferred into the organic phase whereas at 2 M GuHCN only 17% of the protein was transferred. It is known that GuHCN is a more effective denaturant than GuHCl (Lapanje, 1978).

Table A3.1. The forward transfer of lysozyme using various mixtures of DMSO and water into 50 mM AOT/isooctane reverse micelles.

The forward transfer of lysozyme involved mixing equal volumes (5 ml) of an aqueous phase (1 mg/ml lysozyme in 25 mM phosphate buffer, pH 7.0 + DMSO in various ratios + 0.1 M KCl) with an organic phase (50 mM AOT/isooctane). The forward extraction was performed over 15 min and then the samples were centrifuged at 2000 rpm for 5 min. Protein concentration was determined in both phases using A_{280} measurements.

DMSO: Water	% protein transfer to organic phase	% protein left in aqueous phase	Precipitate formation
9:1	0	98.3	No
8:2	7.3	96.1	No
7:3	1.6	5.9	Yes
5:5	97.8	10.0	No
4:6	92.1	1.0	No
3:7	95.9	3.9	No
2:8	94.3	7.0	No
1:9	99.2	3.2	No

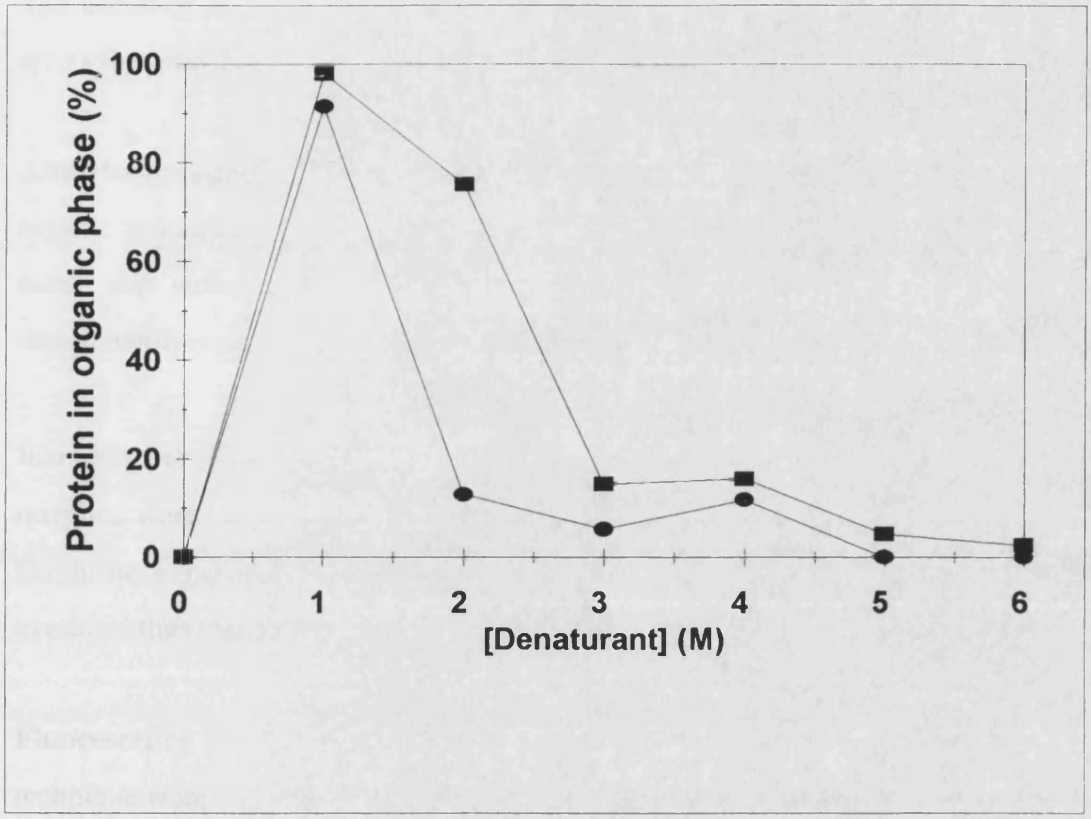


Figure A3.2. The forward transfer of lysozyme with GuHCl and GuHCN

The forward extraction of lysozyme involved mixing equal volumes (5 ml) of 400 mM AOT/isooctane with an aqueous phase (1 mg/ml lysozyme in various concentrations of GuHCl (■) and GuHCN (●) in Tris-HCl, pH 8.7) for 10 min. The samples were then centrifuged at 2000 rpm for 10 min and the protein concentration was determined in the organic phase.

APPENDIX 4

Experimental reproducibility

The accuracy of the methods used throughout this thesis with the experimental errors that may have occurred are included below.

Absorbance spectrophotometry: All protein concentration measurements in the organic and aqueous phases, lysozyme activity assay, Ellman's assay, Folin-lowry assay and surfactant concentration measurements were an average of three determinations each with an error of ± 10%.

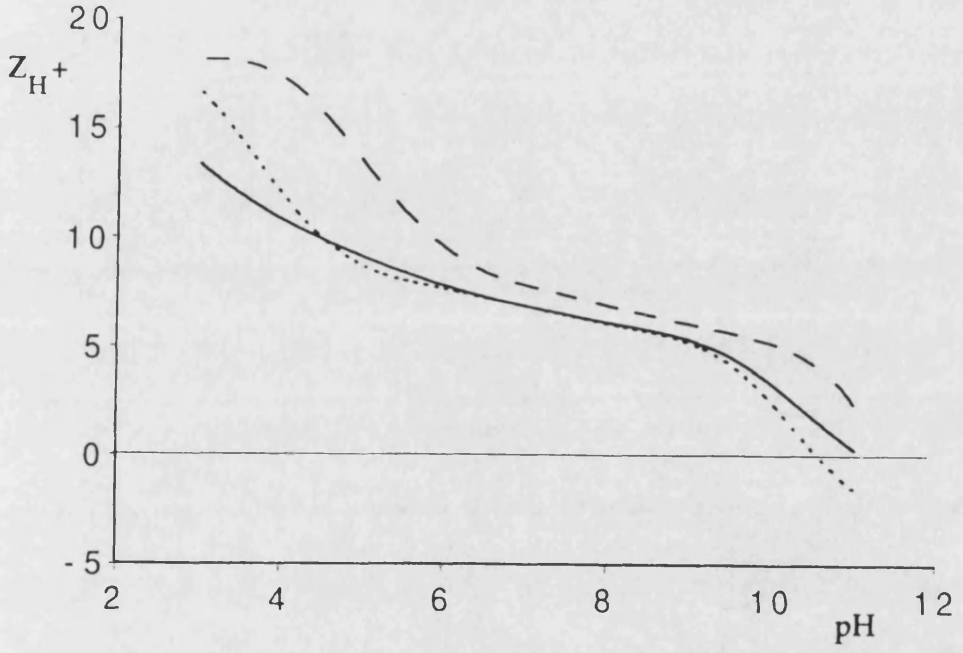
Karl-Fischer titration: The values for the water content inside the reversed micelles were an average of three determinations each with an error of ± 5%. Duplicate experiments were carried out on the water titrations and the results averaged thus the points shown are the average of six separate measurements.

Fluorescence spectroscopy: The conformations of the protein obtained using this technique were an average of three determinations each with an error of ± 5%.

Circular dichroism: Each spectrum shown was an average of three readings each with an error of ± 2% and in all cases the relevant background was subtracted.

APPENDIX 5

Charge distribution of lysozyme as a function of pH



A5.1 Proton titration of lysozyme

Native lysozyme (solid lines) and after denaturation with 6 M GuHCl (dotted lines) or SDS (dashed lines), adapted from Galisteo and Norde (1995). The graph indicates that native lysozyme has a pI of 10.9 compared to values of 10.5 and 11.2 for the GuHCl and SDS denatured forms of the protein.